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Author
O'Riordan, Mary X. D.

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Transcriptional Control of Early B Cell Differentiation

by

Mary X. D. O'Riordan

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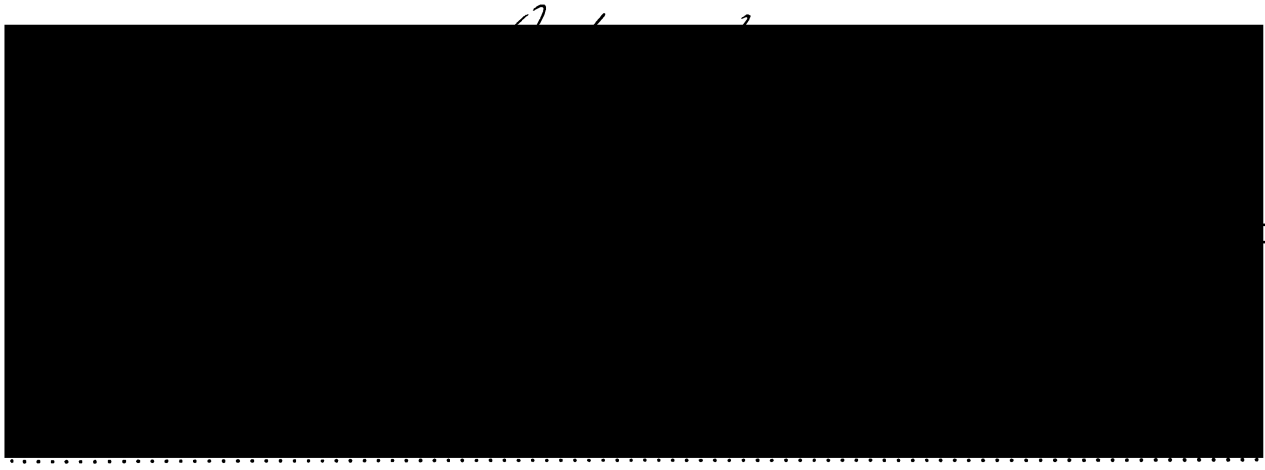

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Dedicated to

Desmond & Phung O'Riordan

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Chapter 3 of this dissertation is a reprint of the material as it appears in *Immunity*, M. Sigvardsson, M. O'Riordan and R. Grosschedl, (1997) 7:25-36. Chapter 4 of this dissertation is a reprint of the material as it will appear in *Immunity*, M. O'Riordan and R. Grosschedl, (1999) *in press*.

Contribution to Described Work

I designed and executed all the experiments in Chapter 2. The work done in Chapter 3 is a publication in *Immunity*, by Mikael Sigvardsson, myself and Rudolf Grosschedl. I contributed data to all figures in Chapter 3 except Figure 6. I helped to design the experiments described in Chapter 3 and helped to write and revise the manuscript, and prepare the figures for publication. The experiments in Chapter 4, and in both appendices that are related to Chapter 4, were solely designed and carried out by myself. I wrote the resulting paper, which will be published in *Immunity*, and oversaw its submission, revision and acceptance. All experimental results shown in Chapter 5 are the result of my work, done as part of a collaboration with Tannishtha Reya and Rudolf Grosschedl.

Transcriptional Control of Early B Cell Differentiation

Mary X. D. O'Riordan

Cell type specific differentiation allows higher organisms to dedicate groups of cells to a specialized function. Transcription factors direct cell type specific differentiation by regulating the expression of tissue specific genes. These genes determine the morphological and functional characteristics of the cell. I have studied the role of three transcription factors, EBF, E2A and LEF-1, in the differentiation of B lymphocytes. EBF and E2A are required at the earliest stage of B cell development. I show that in the B cell lineage, EBF and E2A coordinately regulate the lymphoid specific genes *Pax5*, *mb1*, *Rag1*, *Rag2*, *λ5* and V_{preB}. Furthermore, EBF and E47, a splice variant of the *E2a* gene, can directly and synergistically regulate the transcription of the *λ5* gene. I also find that EBF can directly bind and activate the promoter of the *Pax5* gene. The transcription factor LEF-1, also expressed in early B cells, is a downstream effector of the Wnt/wingless signaling pathway that has been implicated in determining cell fate, and in cell survival and proliferation. I show that LEF-1 is required in pro-B lymphocytes for the normal expression of the *TdT*, *fas*, *N-myc* and *c-myc* genes. Taken together, these observations define a hierarchy of transcriptional regulators that control B lymphocyte differentiation.

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BERKELEY DAVIS IRVINE LOS ANGELES RIVERSIDE SAN DIEGO SAN FRANCISCO SANTA BARBARA SANTA CRUZ

SCHOOL OF MEDICINE

Department of Microbiology and Immunology

SAN FRANCISCO, CALIFORNIA 94143-0414
Fax: (415) 476-8201

May 11, 1999

Mary O'Riordan
Department of Microbiology & Immunology
University of California
San Francisco, CA 94143-0414
phone (415) 476-6954
fax (415) 476-0939
email *mor@itsa.ucsf.edu*

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I am writing to request formal permission from Immunity to reprint the article "EBF and E47 Collaborate to Induce Expression of the Endogenous Immunoglobulin Surrogate Light Chain Genes" (authored by Mikael Sigvardsson, myself and Rudolf Grosschedl) for my doctoral dissertation. The appropriate reference for this article is *Immunity*, Vol. 7, 25-36. University Microfilms requests permission to supply single copies on demand. Thank you for your consideration.

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CHAPTER 1

Introduction

The dedication of cells to a specialized function is of particular importance to multi-cellular organisms. This process of cellular differentiation allows the evolutionary development of molecular mechanisms that are exquisitely suited to benefit the organism in its environment. The program of differentiation often has both extrinsic and intrinsic components. The integration of signals from the extracellular environment provides critical information to the differentiating cell. The intrinsic component of the program consists of a genetic cascade of regulatory factors that specify the characteristic functions of that cell type. By studying the hierarchy of transcriptional regulators that direct gene expression during differentiation, we will understand more about the nature of the cell and its function. This thesis focuses on the transcriptional regulation of early B lymphocyte development by three specific transcription factors: early B cell factor (EBF), E2A and lymphoid enhancer factor-1 (LEF-1).

B Lymphocyte Differentiation

B lymphocyte differentiation is a complex process that is directed towards the production of a successfully rearranged antibody, encoded by the immunoglobulin (Ig) genes (reviewed in Osmond et al., 1998; Reya and Grosschedl, 1998; Rolink and Melchers, 1993). The progression of B cell differentiation can be characterized by the rearrangement status of the immunoglobulin genes. Rearrangement itself is regulated in both a temporal and spatial manner by many different gene products, most notably the

recombinase activating genes, *Rag-1* and *Rag-2* (Fig 1-1) (Mombaerts et al., 1992; Shinkai et al., 1992; Spanopoulou et al., 1994). The sequence of rearrangement starts with the immunoglobulin heavy chain loci (Ig_H) (reviewed in Blackwell and Alt, 1989; Joho et al., 1983). Gene segments of the D and J families are joined together, followed by rearrangement of the V segments to produce a contiguous VDJ_H gene. A VDJ_H gene with a correct reading frame encodes the variable portion of the heavy chain (μ) protein. The successful translation and surface expression of the complete μ protein, in complex with the surrogate light chains, λ5 and V_{preB}, signals the cell to increase rearrangement of the immunoglobulin light chain (κ, λ) locus (Karasuyama et al., 1990; Karasuyama et al., 1996; Tsubata and Reth, 1990). Recombination of the light chain V and J gene segments occurs in a manner similar to heavy chain rearrangement.

As rearrangement proceeds, the B cell exhibits characteristic changes that have been used to define the developmental program. Several types of changes may be observed such as the state of proliferation and changes in gene expression (Fig 1-2). Some differences in gene expression are reflected at the cell surface. Detection of surface antigens using fluorescence conjugated antibodies provides a convenient method for tracking development progression. Importantly, the presence of certain surface antigens may reflect commitment to a particular lineage. The expression of the CD19 surface antigen is considered to be characteristic of the B cell lineage (Rickert et al., 1995).

The lymphocyte lineage derives from the multi-potential hematopoietic stem cell (HSC). Gene targeting technology has been used to show that many transcription factors are required during the progression of the HSC to differentiation into the B cell lineage (Singh, 1996). Some transcription factors are important in the differentiation of multiple lineages, such as PU.1 or Ikaros (Georgopoulos et al., 1994; Scott et al., 1994). Other transcription factors seem to contribute exclusively to one lineage within the hematopoietic system, such as EBF or BSAP, encoded by the *Pax5* gene, which are required only for the development of B cells (Lin and Grosschedl, 1995; Urbanek et al., 1994). Interestingly, few of these factors are truly lineage specific. EBF is expressed in adipose tissue, as well as in the brain and olfactory neurons (Hagman et al., 1993). *Pax5* is also expressed in the brain (Adams et al., 1992). What factor or combination of factors irrevocably defines the fate of a B lymphocyte rather than a neuron is a compelling question.

Mechanisms of Transcriptional Regulation

Transcription factors play a critical role in cell fate determination and specification. Every cell contains basic transcriptional machinery, consisting of a promoter recognition component, such as TBP, coupled with RNA polymerase holoenzyme (Fig. 1-3A) (reviewed in Burley and Roeder, 1996; Zawel and Reinberg, 1995). This basal machinery requires the assistance of transcriptional regulators for the expression of tissue specific genes. This requirement imposes

spatial and temporal limits on gene expression dependent on the continued activity of transcriptional regulators. Transcription factors may stimulate gene expression by a number of diverse mechanisms. First, activators, such as the glucocorticoid receptor (GR), may recruit chromatin remodeling complexes that make regulatory regions more accessible to other factors (Paranjape et al., 1994; Zaret and Yamamoto, 1984). Second, transcription factors can nucleate a complex of other transcription factors and co-activators, either through interaction or, in the case of LEF-1, by sharply bending the DNA to allow distant sites to be juxtaposed (Giese et al., 1995). Third, binding of one transcription factor may augment the DNA binding capability of another, as has been shown for the proteins, Pip and E47 (Nagulapalli and Atchison, 1998). Fourth, specific factors may interact directly with components of the basal transcription machinery, as shown for SP-1 and dTAF$_{II}$110, part of the TFIID complex (Gill et al., 1994). Complex promoters that contain binding sites for many different factors may require the activity of different types of transcription factors, for example, one to recruit chromatin remodeling complexes, and another to interact with the basal transcription machinery. These factors may synergistically regulate the promoter such that the activity of both is much greater than the activity of either alone. Activation of the low density lipoprotein receptor (LDLR) promoter requires the activity of both SP-1 and SREBP-1α (Naar et al., 1998). In order to understand the contribution of a transcriptional activator in a

given cell type, it is important to define both the requirements of the activator to perform its function, as well as its downstream targets.

Early B cell Factor

Early B cell factor (EBF) is a prototypic transcriptional regulator expressed in all stages of developing B cells until the point of terminal differentiation into a plasma cell. EBF was independently identified in olfactory neurons as Olf-1 (Wang and Reed, 1993). EBF belongs to a family of transcription factors, including the *Drosophila* homolog, collier, the *C. elegans* homolog, unc-3, as well as several mammalian homologs (Crozatier et al., 1996; Dubois et al., 1998; Garel et al., 1997; Prasad et al., 1998; Wang et al., 1997). While the different family members are expressed in a wide variety of tissues, most have been implicated in tissue specific differentiation. Mice lacking a functional *Ebf* gene, have a severe and early block in B cell development (Lin and Grosschedl, 1995). Recent studies have also revealed defects in differentiation of specific groups of neurons in the brain of $Ebf^{-/-}$ mice (S. Garel and P. Charnay, personal communication). Interestingly, a partial phenotype can also be observed in $Ebf^{+/-}$ mice, where the B cell compartment is reduced by fifty percent (Lin and Grosschedl, 1995).

The EBF/Olf-1/collier family of transcription factors exhibits some unusual biochemical properties (Hagman et al., 1995). EBF has a large DNA binding domain, of approximately 200 amino acids, that contains a unique zinc coordination motif ($HX_3CX_2CX_5C$) (Fig. 1-3B). This domain is necessary and

sufficient to bind the consensus sequence 5'ATTCCCnnGGGAAT, although EBF binds DNA much more efficiently as a dimer. Most natural EBF binding sites deviate significantly from the consensus site. Curiously, on consensus binding sites, EBF can form dimers through its DNA binding domain, although degenerate sites require the more C-terminal dimerization motif. EBF homodimerizes through two α-helical domains that resemble the helix-loop-helix (HLH) domains of the family of bHLH transcription factors. The serine/threonine rich transcriptional activation domain is contained at the C-terminal end of the protein. Unlike many activators, EBF does not activate strongly from multimerized EBF binding sites (M. O'Riordan, R. Grosschedl, unpublished observations). The *mb-1* promoter, that contains the binding site originally used to characterize EBF, has sites for several other transcription factors as well, including SP1 and Ets (Hagman and Grosschedl, 1992; Travis et al., 1991). EBF may require the activity of other transcription factors in order to perform its transactivating function.

E2A

The *E2a* gene encodes two transcription factors, E12 and E47, which through alternative splicing, differ in their basic helix-loop-helix (bHLH) domains (Fig 1-3C). E2A, and other members of the *daughterless* gene family, bind the consensus sequence 5' CANNTG (reviewed in Bain and Murre, 1998; Murre et al., 1994). These proteins are ubiquitously expressed, but

heterodimerize with tissue specific bHLH factors such as MyoD (Lassar et al., 1991). Thus, they have been implicated in the differentiation of many different cell types, despite their widespread expression pattern. E2A forms a unique protein in the B cell lineage, termed B Cell Factor-1 (BCF-1) (Shen and Kadesch, 1995). BCF-1 consists of an E47 homodimer, possibly stabilized by intermolecular di-sulfide bonds (Benezra, 1994). Protein dephoshorylation may also play a role in the formation of the homodimer, although the regulation of BCF-1 is still incompletely understood (Sloan et al., 1996).

Surprisingly, the fetal development of *E2a*$^{-/-}$ mice is apparently normal, however, development of the B cell lineage is completely blocked (Bain et al., 1994; Zhuang et al., 1994). The stage at which B cell differentiation is arrested in *E2a*$^{-/-}$ mice is similar to that observed in the *Ebf* deficient mice. Studies in which an E12 or E47 transgene (E12T, E47T) was added to the *E2a* null background indicated that E12 and E47 are not completely redundant (Bain et al., 1997). *E2a*$^{-/-}$*;E47T* mice were able to support B cell development through all stages, although significantly reduced in number. *E2a*$^{-/-}$*;E12T* mice did not support further B cell development, but did allow commitment or survival of greater numbers of B cells before the developmental defect. Hence, both E12 and E47 contribute uniquely to differentiation of the B cell lineage.

The activity of E2A is subject to negative regulation by proteins of the Id family (Wilson et al., 1991). These factors contain an HLH domain, and can therefore heterodimerize with members of the bHLH family. However, both

basic domains of the dimer are required for DNA binding, so Id:E2A heterodimers are unable to bind DNA or activate transcription. Id-1 is expressed in the B cell lineage, but expression progressively decreases through the early stages of development (Saisanit and Sun, 1995; Simonson et al., 1993). The ratio of bHLH to HLH factors in the B cell lineage is critical, as lowering the genetic dose of E2A, or the related genes, E2-2 or HEB results in fewer B lymphocytes (Zhuang et al., 1996). Similarly, an increase in the amount of Id-1, as seen in an Id-1 transgenic mouse, negatively affects the generation of B cells (Sun, 1994).

Binding sites for the E2A proteins are found in many B cell specific regulatory regions, notably the immunoglobulin intronic enhancer (E_μ) (Henthorn et al., 1990; Murre et al., 1989). These sites, bound by BCF-1, are known to be important for the function of the enhancer. E47, overexpressed in non-B cell lines, has been shown to upregulate transcription of I_μ, the sterile promoter contained within the Ig intronic enhancer (Choi et al., 1996; Schlissel et al., 1991). Transcription of the lymphoid specific genes *Rag-1*, *TdT* and *Oct-2* were also increased. It is thought that the overexpression of E47 allows the formation of some E47 homodimers, perhaps titrating out the regulatory activity that normally prevents the formation of BCF-1 in non-B cells. Until recently, little was known about the targets of E12 in B cell differentiation. Expression of E12 in a macrophage-like cell line upregulates both *Ebf* and *Pax5* expression (Kee and Murre, 1998). These data suggest that E12 plays an especially important role in the early stages of B cell differentiation by upregulating key transcriptional

activators. However, the macrophage-like cell line used, 70Z/3, was derived from a B cell line, so it is difficult to conclude that E12 is sufficient for the upregulation of these B cell specific genes. The possibility remains that another important regulator of B cell development may still be expressed in the 70Z/3 macrophage cell line. Nevertheless, it is clear that both E12 and E47 regulate target genes that are required for normal B cell differentiation.

Pax5 (BSAP)

BSAP, the transcription factor encoded by the *Pax5* gene, is a critical factor in the differentiation of B lymphocytes (Urbanek et al., 1994). *Pax5* is a member of the *Pax* gene family, and contains a paired domain responsible for DNA binding, and a homeodomain (reviewed in Wehr and Gruss, 1996). Members of this family are also found in *C. elegans*, *Drosophila* and sea urchin, with paired domains that are essentially identical in sequence and function. *Pax5* is expressed throughout B cell development, but is downregulated upon terminal differentiation (Fig. 1-2) (Adams et al., 1992). This pattern of expression mirrors that of *Ebf*. *Pax5*$^{-/-}$ mice exhibit a complete arrest of differentiation slightly later than that observed in the *Ebf*$^{-/-}$ mice or *E2a*$^{-/-}$ mice (Urbanek et al., 1994). These observations are consistent with the hypothesis that *Pax5* is a target of EBF. The regulation of *Pax5* is not well understood. Two alternative start sites have been identified in both human and mouse which, through alternative splicing, encode

almost identical proteins with a few amino acids differing at the N-terminus (Busslinger et al., 1996). The upstream start site, designated Exon 1A, appears to be used exclusively in the B cell lineage. Exon 1B is expressed in the central nervous system and testis, as well as in B lymphocytes.

Much work has been done to identify BSAP target genes; however, the results have somewhat controversial. Promoter analyses of many genes including *CD19*, *mb-1*, *blk*, *XBP-1*, *λ5* and V_{preB}, all expressed during B cell development, have identified functional BSAP binding sites (reviewed in Neurath et al., 1995). Surprisingly, recent experiments using *Pax5*$^{-/-}$ pro-B cells have shown that only the *CD19*, *mb-1*, *N-myc* and *Lef-1* genes are likely to be direct targets of BSAP transcriptional activation (Nutt et al., 1998). The importance of the BSAP binding sites in the promoters of other genes, such as *λ5* and V_{preB}, is unclear. The same study also identified the gene *PD-1* as a target of transcriptional repression by BSAP, indicating that this factor plays a complex role in differentiation.

LEF-1

Lymphoid enhancer factor-1 (LEF-1), a target of BSAP in B lymphocytes, is expressed in many different tissues. During embryonic development, LEF-1 is necessary for organogenesis in the regulation of mesenchymal-epithelial interactions (Kratochwil et al., 1996; van Genderen et al., 1994). The activity of the LEF/T-cell factor (TCF) family of transcription factors can be modified by

interaction with another protein, β-catenin (Behrens et al., 1996; Hsu et al., 1998; Huber et al., 1996; van de Wetering et al., 1997). β-catenin participates in the Wnt/Wingless signaling pathway, implicated in many developmental processes such as axis formation and cell fate determination (Fig. 1-4A) (Wodarz and Nusse, 1998). This pathway translates external cues into a specific transcriptional program, allowing developing cells to respond to their immediate environment. Several members of the Wnt family are expressed in hematopoietic stromal tissues, such as the fetal liver, thymus and bone marrow although little is known about their function in B or T cell differentiation (T. Reya, M. O'Riordan and R. Grosschedl, unpublished observations) (Van Den Berg et al., 1998). During T cell development, LEF-1 or TCF-1 is required in order for T cells to become $CD4^+CD8^+$(Okamura et al., 1998; Verbeek et al., 1995). The T cell receptor alpha (TCRα) enhancer has been used as a model to characterize the biochemical functions of LEF-1 (Fig. 1-4B). The binding of LEF-1 to DNA induces a sharp bend that allows the juxtaposition of non-adjacent factor binding sites (Giese et al., 1992). Furthermore, LEF-1 acts to recruit the co-activator, ALY, which may be important for the nucleation of the higher order protein complex required for TCRα activation (Bruhn et al., 1997). This function of LEF-1 is thought to be independent of the Wnt pathway.

In B cells, LEF is expressed during pro-B cell development, along with another member of the TCF family, TCF-4 (T. Reya, E. Devaney and R. Grosschedl, unpublished observations; Korinek et al., 1998). *Lef*$^{-/-}$ mice contain

mature B lymphocytes in the periphery, but studies of early B cell differentiation reveal that development is peturbed (T. Reya and R. Grosschedl, unpublished data). The role of the Wnt pathway and LEF-1 in B cell development is currently under investigation.

The Thesis

In this thesis, I present my research into the network of transcriptional activators that control early B cell differentiation. Early studies on the function of EBF suggested that EBF requires interaction with other activators. In chapter 2, I show that an evolutionarily conserved region of EBF, with no assigned biochemical function, contributes to transcriptional activation. I also investigate a possible interaction between EBF and another transcription factor, E47.

In Chapter 3, I describe experiments in which EBF and a B cell specific form of E2A are expressed in a non-B cell line. These results show that EBF and E2A act synergistically to upregulate the endogenous B cell specific genes, $\lambda 5$ and V_{preB}. These genes encode the immunoglobulin surrogate light chains. Furthermore, functional binding sites for EBF and E2A in the $\lambda 5$ promoter are identified by electrophoretic mobility shift assay (EMSA) or methylation interference analysis. EBF and E2A can synergistically activate the $\lambda 5$ promoter in transient transfection assays, indicating that these factors are likely to be the only B cell specific activities required.

Chapter 4 demonstrates a genetic interaction between EBF and E2A in B lymphocyte differentiation. I define the stage in development at which these proteins are required at wildtype levels. An extensive analysis of gene expression in *Ebf*$^{+/-}$*E2a*$^{+/-}$ B cells reveals a number of downstream target genes, including *Pax5*, *Rag-1* and -2, *Lef-1*, *λ5* and V_{preB}. To demonstrate the relationship between EBF and E2A and their earliest known genetic target, *Pax5*, I cloned approximately two kilobases of *Pax5* promoter sequence. I demonstrate using both EMSA and transient transfections that *Pax5* is a direct target of EBF.

Chapter 5 describes an analysis of gene expression in *Lef*$^{-/-}$ B lymphocytes. I find that several genes are differentially expressed in *Lef* deficient fetal liver compared to wildtype, notably *TdT*, *Fas*, *N-myc* and *c-myc*. I also describe an experiment in which cultured wildtype or *Lef*$^{-/-}$pro-B lymphocytes are deprived of growth factor and analyzed for changes in expression. Under these circumstances, *Lef*$^{-/-}$pro-B cells considerably upregulate the expression of the *Tcf-4* and *N-myc* genes. These data are consistent with a role for LEF/TCF family members in translating growth and survival signals during B cell development. Thus, in total, we have linked a cascade of regulatory factors that direct multiple aspects of B cell differentiation from transcription to signaling and survival.

The final chapter, Chapter 6, summarizes the conclusions from the entire thesis and integrates them into a view of early B cell differentiation that includes current knowledge in the field.

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Figure 1-1.

Immunoglobulin rearrangement and surface antigen expression during B lymphocyte differentiation.

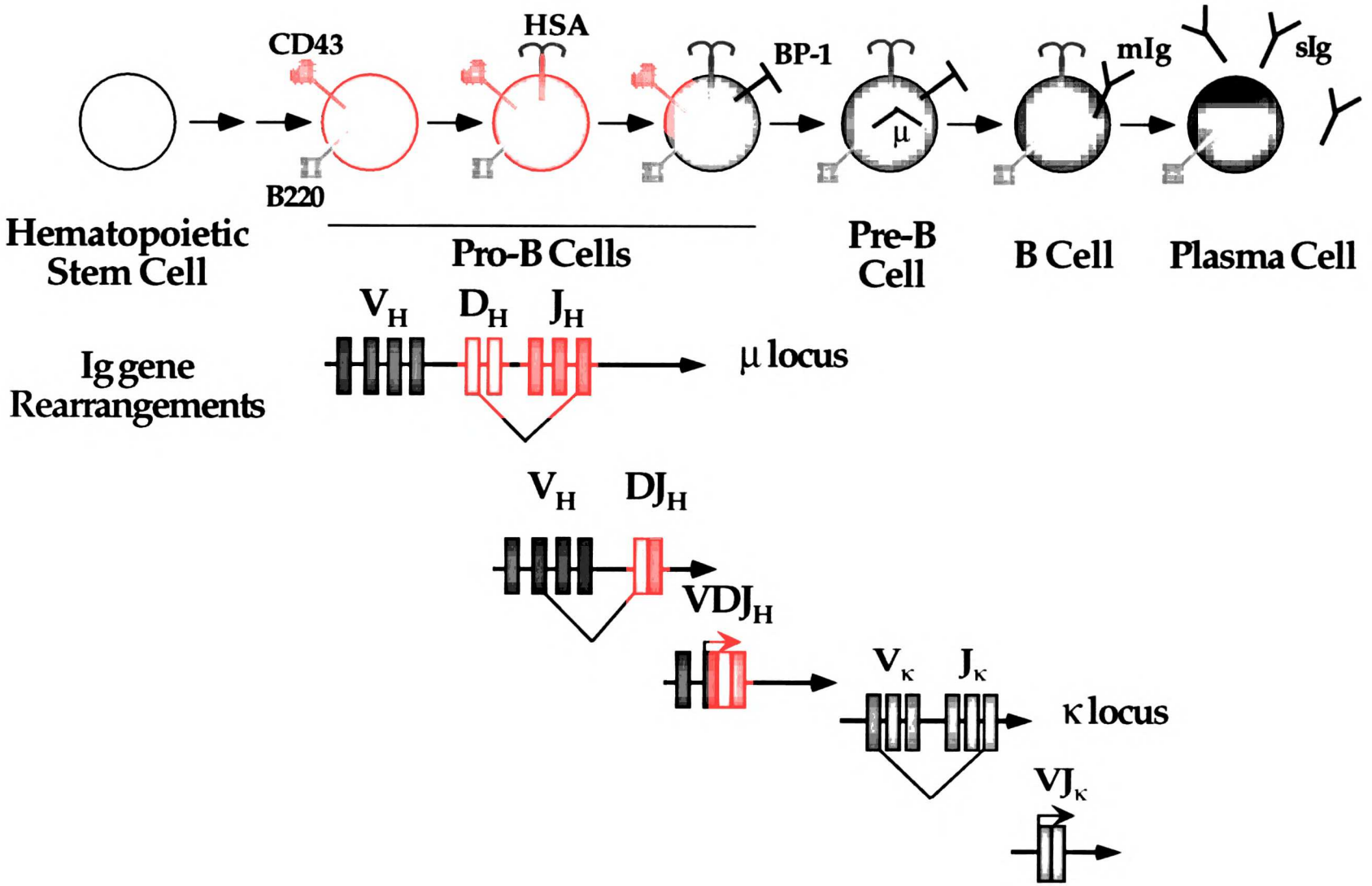

CD43
HSA
BP-1
mIg
sIg
B220
μ
Hematopoietic Stem Cell
Pro-B Cells
Pre-B Cell
B Cell
Plasma Cell
Ig gene Rearrangements
V_H D_H J_H
μ locus
V_H DJ_H
VDJ_H
V_κ J_κ
κ locus
VJ_κ

Figure 1-2.

Expression pattern of transcription factors and lymphoid specific genes during B lymphocyte differentiation.

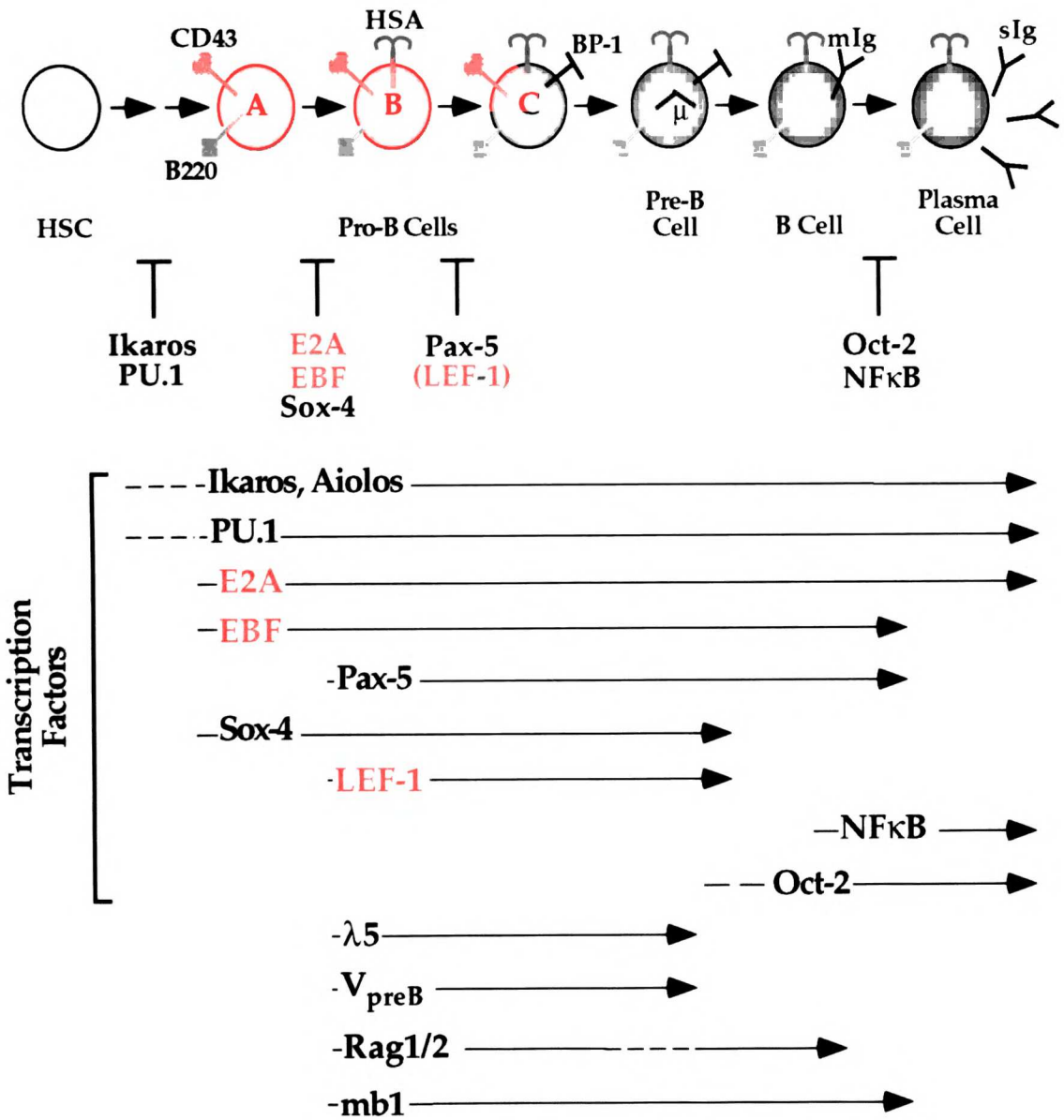

CD43
HSA
BP-1
mIg
sIg
A
B
C
μ
B220
HSC
Pro-B Cells
Pre-B Cell
B Cell
Plasma Cell
Ikaros
PU.1
E2A
EBF
Sox-4
Pax-5
(LEF-1)
Oct-2
NFκB
Transcription Factors
Ikaros, Aiolos
PU.1
E2A
EBF
Pax-5
Sox-4
LEF-1
NFκB
Oct-2
λ5
V preB
Rag1/2
mb1

Figure 1-3.

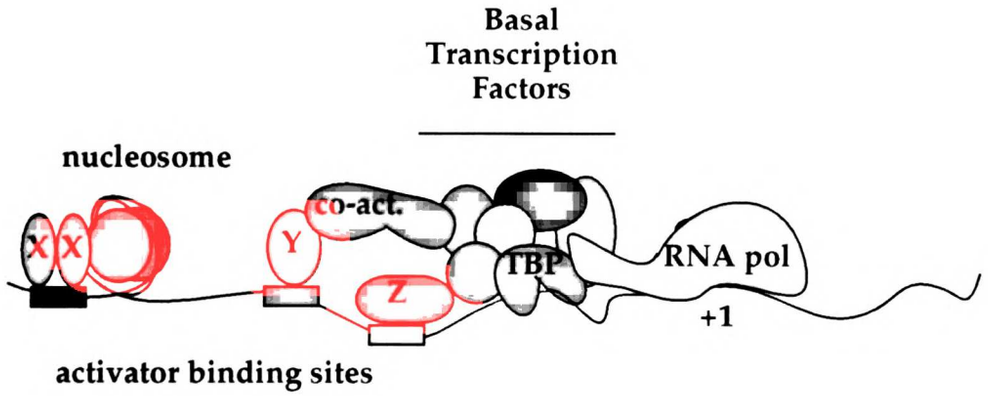
Transcriptional Activation.

(A) A model of basal transcription machinery and activated transcription. Objects in blue represent basal transcription factors, such TFIIA, B, D, E and F. TFIID is a multi-protein complex that includes TBP (green) and TBP-associated factors (TAFs). Transcriptional activators (shown as X, Y and Z) may act by changing chromatin structure, displacing specific repressor proteins, recruiting co-activator proteins or by directly contacting the basal transcription complex.

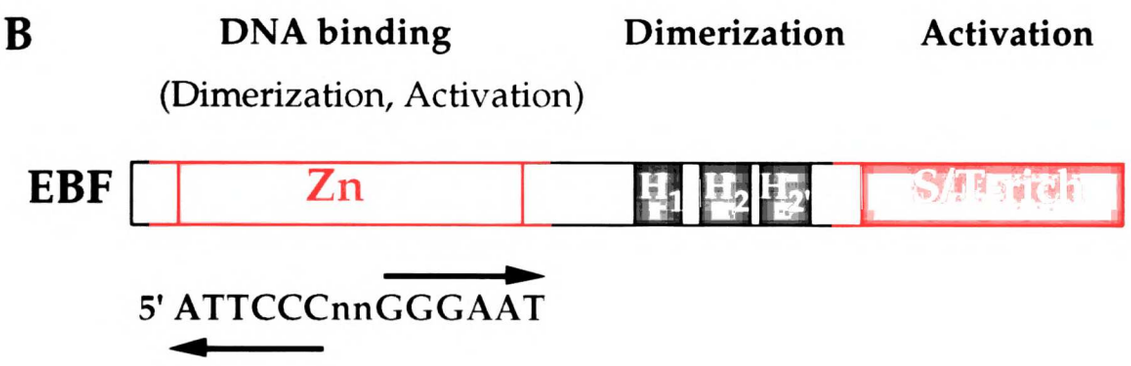
(B) Schematic representation of the transcription factor, EBF. Functional domains are shown as colored rectangles. The activation domain of EBF consists of a serine/threonine rich region. The consensus DNA binding site is shown below the DNA binding domain.

(C) Schematic representation of the transcription factor, E2A. Functional domains are shown as colored rectangles. The consensus DNA binding site, termed E-box, is shown below the DNA binding domain.

A


Basal
Transcription
Factors
nucleosome
X
X
co-act.
Y
Z
TBP
RNA pol
+1
activator binding sites

B


DNA binding
(Dimerization, Activation)
Dimerization
Activation
EBF
Zn
H1
H2
H2'
5' ATTCCCnnGGGAAT

C

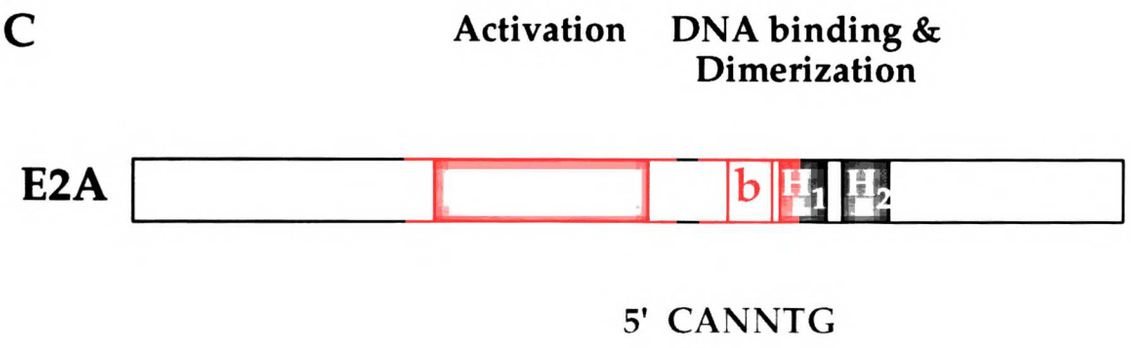

Activation
DNA binding &
Dimerization
E2A
b
H1
H2
5' CANNTG

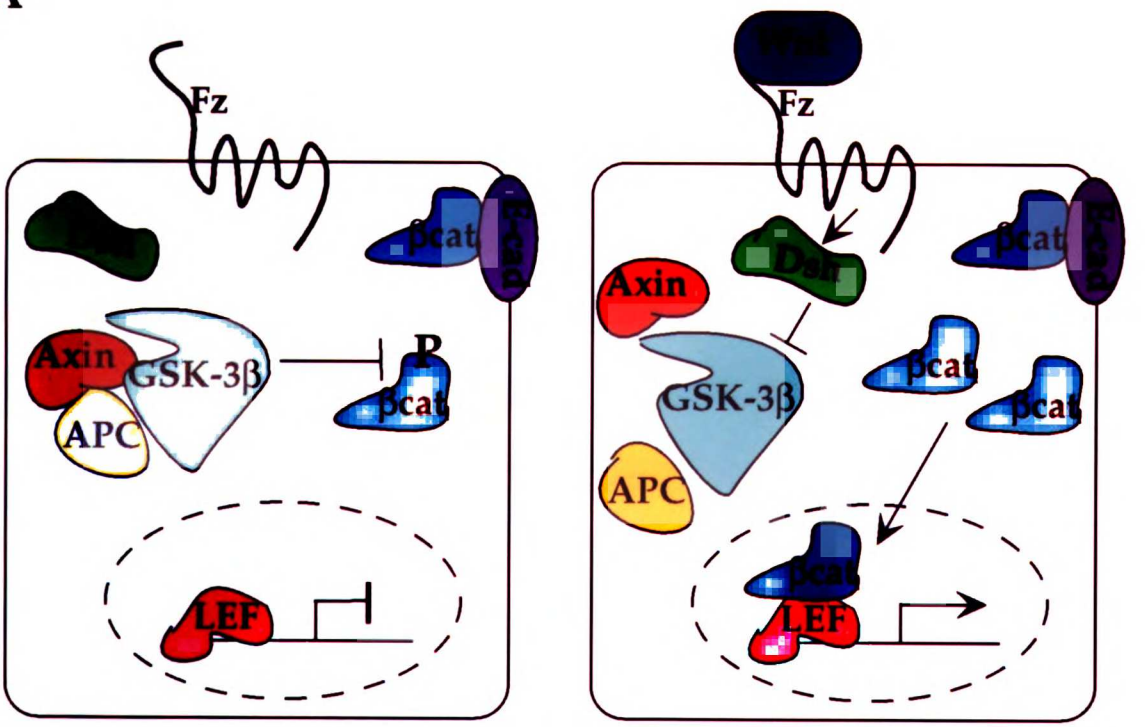
Figure 1-4.

LEF-1 and the Wnt Signaling Pathway.

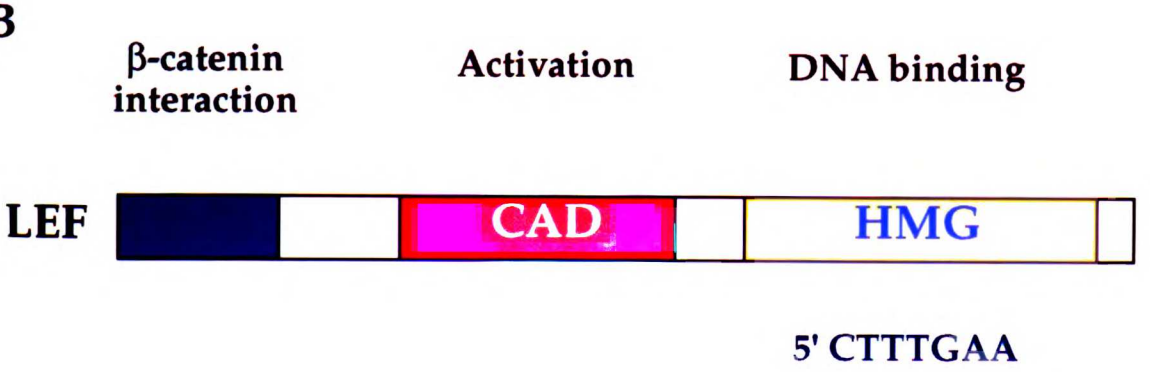
(A) A model of the mammalian Wnt/Wingless signaling pathway. When the Frizzled receptor (Fz) is disengaged, an inhibitory complex containing Axin, Adenomatous Polyposis Coli (APC) and Glycogen Synthase Kinase-3β (GSK-3β) phosphorylates β-catenin (β-cat P). Phosphorylated β-catenin is rapidly degraded and does not enter the nucleus. Upon Wnt signaling, a signal is transduced through the Disheveled protein (Dsh) that results in relief of inhibition by GSK-3β. β-catenin is not phosphorylated and rapidly accumulates. Free β-catenin translocates to the nucleus and activates transcription in conjunction with members of the LEF/TCF family of transcription factors.

(B) Schematic representation of the transcription factor, LEF-1. Functional domains are shown as colored rectangles. LEF-1 contains an unusual context dependent activation domain (CAD) and a high mobility group (HMG) type DNA binding domain. The consensus DNA binding site is shown.

A


Fz
βcat
Axin
GSK-3β
APC
P
βcat
LEF
Fz
Dsh
Axin
βcat
βcat
GSK-3β
APC
βcat
LEF

B


β-catenin interaction
Activation
DNA binding
LEF
CAD
HMG
5' CTTTGAA

CHAPTER 2

A Domain of EBF Containing the HLH-like Motif Contributes to Transcriptional Activation

Abstract

Early B cell factor (EBF) is part of a highly conserved family of transcription factors implicated in the differentiation of many different cell types. Here we investigate the relationship between EBF and its Drosophila homolog, collier, in order to understand more about the function of EBF in B cells. Collier binds DNA more efficiently than EBF, and can transactivate from a mammalian EBF binding site as well as EBF itself. We demonstrate that the deletion of a domain conserved between EBF and collier, with no assigned biochemical function, can affect the ability of EBF to activate transcription from a complex promoter. We investigate the ability of EBF to interact with a candidate partner, E47. Taken together, these data suggest that EBF interacts with another protein, not E47, that contributes to promoter activation.

Introduction

Genetic sequences encoding modular protein domains are often conserved throughout evolution if the function of that domain is necessary. Comparison of homologous sequences between two divergent species has been a useful tool to highlight which parts of a protein have particularly important functions. Members of the EBF family of transcription factors exist in species that are as evolutionarily divergent as the fruit fly and the mouse (Crozatier et al., 1996; Dubois et al., 1998; Hagman et al., 1993; Prasad et al., 1998; Wang and Reed, 1993). At the amino acid level, known functional domains of these proteins, such as the 202 amino acid DNA binding domain, may be as high as 88% identical, or 94% conserved (Crozatier et al., 1996). The EBF/Olf-1/collier proteins appear to function in diverse tissues, although their biochemical function must be conserved.

EBF is expressed in B lymphocytes, olfactory neurons, adipose tissue and brain (Hagman et al., 1993; Wang and Reed, 1993). The most well understood function of EBF is in B cell development, which has no analogy in *Drosophila*. In contrast, collier is expressed in a mitotic domain of the *Drosophila* embryo that later becomes part of the adult head (Crozatier et al., 1996). Although there is no apparent connection between the mandibular segment anlage and early B lymphocytes, it is most likely that EBF and its homolog participate in a conserved pathway that contributes to the differentiation of many different cell

types. In order to define the importance of this pathway, its participants must be identified. Thus, we are interested in what proteins may interact with EBF to activate transcription.

EBF contains three known functional domains: a large DNA binding domain, an alpha-helical homodimerization domain and a C-terminal activation domain (Fig. 2-1) (Hagman et al., 1995). The DNA binding domain of EBF has also been shown to mediate homodimerization on consensus binding sites, but not degenerate binding sites. Collier has similar organization with an interesting exception: part of the alpha-helical homodimerization domain is not present (Crozatier et al., 1996). Furthermore, a putative helix-loop-helix region for which there is no known function, remains intact and remarkably well conserved. The helix-loop-helix motif mediates protein-protein interactions among many different proteins, most notably, those of the family of basic helix-loop-helix (bHLH) transcription factors (Murre et al., 1994). These factors which include, c-myc, E2A and *Drosophila* daughterless, can homo- or hetero-dimerize through the HLH domain (Murre et al., 1989; Murre et al., 1989).

E2A, a transcription factor of the bHLH family, is expressed in lymphoid tissues at a similar stage as EBF (Jacobs et al., 1993; Jacobs et al., 1994). E2A encodes two alternate splice forms, E12 and E47, that differ in the bHLH region (Aronheim et al., 1993; Murre et al., 1989). Both E12 and E47 are expressed in B lymphocytes, and E47 uniquely forms homodimers in the B lineage (Aronheim et al., 1993; Shen and Kadesch, 1995). E2A binding sites or E-boxes exist in the

promoters of many different B cell specific genes (Murre et al., 1994). In addition, mice lacking EBF or E2A exhibit a similar B cell phenotype, possibly suggesting either a biochemical or genetic interaction between these two transcriptional regulators (Bain et al., 1994; Lin and Grosschedl, 1995; Zhuang et al., 1994).

Here we investigate the importance of a functional domain conserved between EBF and collier that may be involved in protein-protein interaction. We look for such an interaction by overexpression and immunoprecipitation of EBF in COS7 cells. We also test a possible interaction between the two transcription factors, EBF and E47.

Results

Collier binds DNA more efficiently than EBF.

In order to understand the significance of the conservation between EBF and collier, we performed experiments to define the ability of collier to function in mammalian systems. Collier and EBF were translated *in vitro* in reticulocyte lysates, and used in electrophoretic mobility shift assays (Fig. 2-2). EBF binds most efficiently as a dimer, to DNA sequences containing a palindromic consensus site (*pal*): 5' ATTCCCnnGGGAAT (Hagman et al., 1995; Travis et al., 1993). However, most natural sites deviate significantly from this sequence, therefore we also tested the ability of these two factors to bind a physiological EBF binding site found in the *mb-1* promoter (*mb-1*) (Hagman et al., 1993).

Lastly, we tested the binding activity of collier and EBF to a non-optimal EBF site that consists of a palindromic sequence where the two half sites are separated by four nucleotides (*pal+4*): 5′ ATTCCCnnnnGGGAAT. EBF binds poorly if at all to this site, presumably due to physical contraints imposed by dimerization of the protein. In an experiment using these three labeled DNA sequences as probes, we found that in every case collier bound more efficiently than EBF (Fig 2-2B). Most surprisingly, collier seemed to bind fairly well to the *pal+4* sequence, resulting in a mobility shift similar to that of a dimer. However, collier is missing part of the amino acid sequence that is thought to be important for homodimerization of EBF. Furthermore, collier seems to consistently bind as a dimer, not as a monomer, based on its mobility in electrophoretic mobility shift assays. It is possible that collier may be using the weaker dimerization capability of the large DNA binding domain, demonstrated for EBF, and may be less physically constrained than EBF which uses the two C-terminal alpha-helices to homodimerize. It was not previously thought that either of EBF's helix-2 like (H-2 and H2′) sequences could participate in binding with another factor while in the homodimeric state. Since collier can still dimerize, it may be possible for EBF to maintain homodimerization capability, required for DNA binding, through the DNA binding domain, while interacting with other transcription factors through the helix-loop-helix domain.

Collier can transactivate similarly to EBF in transient transfection assays

In order to test whether the transactivation function of collier was equivalent to that of EBF, we performed transient transfection assays using the *mb-1* promoter. *Mb-1*, which encodes one of the signaling components of the B cell receptor, has a complex promoter in which binding sites for EBF, BSAP, Sp-1 and Ets have been found. EBF activates more strongly from this complex promoter than from multimerized EBF sites. A wildtype or mutant *mb-1*-OVEC reporter construct was transfected into HeLa cells along with increasing amounts of EBF or collier (Fig. 2-3). The OVEC reporter encodes the TATA box and coding sequence of the rabbit β-globin gene (Westin et al., 1987). Transcription can be assayed by annealing a labeled oligonucleotide to the β-globin transcripts contained in the transfected cell lysate, and degrading the non-annealed, or unprotected, transcripts with nucleases. A control construct, containing partial OVEC sequence, can be detected with the same probe allowing evaluation of transfection efficiency. In this assay, collier activated transcription equally well or better than EBF, correlating with the ability of collier to bind DNA more efficiently. Importantly, collier is able to function as a transactivator at the complex *mb-1* promoter, normally active in B cells, in the same manner as EBF.

A partial deletion of the EBF helix-loop-helix sequence decreases transcription.

As we were able to show that collier acted as well or better than EBF in both DNA binding and transactivation, we reasoned that conserved regions of the protein likely participated in an important function. Most regions of EBF

have been biochemically characterized by deletion mapping. Thus, domains of EBF that contribute to DNA binding, homodimerization and transcriptional activation are known. The intervening sequence between the DNA binding domain and the first homodimerization helix (amino acids 251 to 378) does not have a known function, but appears to be well conserved. Therefore, a previously characterized deletion of EBF was used in transient transfection assays to look for a possible contribution to transactivation. This deletion construct, EBFΔ296-367, leaves intact the DNA binding, dimerization and activation domains, but removes the helix-1 sequence. Previous studies using *in vitro* translated EBF showed that EBFΔ296-367 did, in fact, still bind DNA as a homodimer with similar affinity to wildtype EBF (Hagman et al., 1993). EBFΔ296-367 and wildtype EBF were transiently tranfected into a plasmacytoma line, S194 (Fig. 2-4). Transcription of the co-transfected wildtype OVEC reporter was then assayed by S1 nuclease protection. S194 is B lymphoid in origin, and thus is likely to contain many B cell specific factors, but no longer expresses endogenous EBF. Quantitation of the expression of the OVEC reporter and the OVEC control plasmid by phoshorimager revealed a three- to four-fold decrease in transcriptional activation by EBFΔ296-367. We therefore conclude, that the conserved region of EBF between the DNA binding and homodimerization domain contribute to the transactivation function of EBF, possibly through interaction with another transcription factor or co-activator.

This conserved domain, between the DNA binding and the transactivation domains, was fused to the Gal4 DNA binding domain and used as bait in a two-hybrid screen to identify potential EBF interacting proteins. The screen was unsuccessful due to overwhelming numbers of positives that survived every false positive test. Sequencing of a few clones revealed that at least some of the clones were chaperone-like proteins, possibly indicating that the protein was misfolded. A one-hybrid screen using the DNA binding domain of EBF with the helix-loop-helix, but not the transactivation domain, would likely yield better results.

EBF interacts with a 27 kD protein in COS7 cells

In another approach to identifying interacting proteins, we generated an epitope tagged version of EBF in order to perform co-immunoprecipitation experiments, as no high affinity EBF anti-sera is available. Sequence encoding a small bacterially derived peptide (T7) was cloned in-frame onto the C-terminus of *Ebf*. The recombinant protein bound DNA indistinguishably from wildtype *in vitro* translated EBF, and was supershifted by both an anti-T7 antibody and anti-EBF anti-sera (Fig. 2-5A). In transient transfection experiments, EBF-T7 transactivated as well as the wildtype EBF (data not shown). EBF-T7 and EBFΔ296-367-T7 were transiently overexpressed in COS7 cells. After 36 hours, the transfected cells were metabolically labeled with ^{35}S-methionine for two

hours and harvested. Anti-T7 monoclonal antibody was added to the ^{35}S-methionine labeled lysates to immunoprecipitate EBF (Fig. 2-5B). EBF-T7 and EBFΔ296-367-T7 were expressed abundantly, and multiple degradation products were observed. A protein of approximately 27 kD was also observed co-immunoprecipitating with both EBF-T7 and Δ296-367-T7. To distinguish between degradation products and potential interacting proteins, a similar transfection was done without metabolic labeling and the lysates submitted to immunoprecipitation and Western blotting by anti-T7/anti-mouse alkaline phosphatase (data not shown). Multiple C-terminal fragments (the end containing the T7 tag) were apparent on the Western blot, however, none migrated at a rate consistent with a 27 kD protein. N-terminal fragments of EBF would not be present in the immunoprecipitation and furthermore, would not be recognized by the anti-T7 antibody in the Western blot analysis. The low stoichiometry of the 27 kD protein to EBF may be due to the high expression of the transfected proteins in COS7 cells directed by the SV40 origin contained in the plasmids. We infer from these experiments that a 27 kD protein found in COS7 cells interacts with EBF, and this interaction does not require helix 1 of the helix-loop-helix motif. Similar experiments were also attempted in B cell lines, however, low efficiency of transient transfection and abundant amounts of endogenous EBF resulted in experiments characterized by a low signal to noise ratio. More conclusive experiments will require the use of higher immunoaffinity reagents, such as monoclonal antibodies.

EBF does not interact with E47 in transient transfection assays.

While looking for interaction partners in COS7 cells and B cells, we also decided to look for an interaction with a likely candidate regulatory partner. The similarity of the EBF conserved helix-loop-helix sequence to the *daughterless* family of bHLH proteins, led us to look for a possible interaction with the transcription factor, E47. E47, a splice form of the *E2a* gene, homodimerizes uniquely in the B lymphocyte lineage (Shen and Kadesch, 1995). We considered E47 to be a reasonable candidate for interaction with EBF, since they are expressed in similar stages of B cell differentiation. Furthermore, *Ebf* and *E2a* deficient mice have a very similar B lineage developmental arrest. We therefore overexpressed T7-tagged EBF, and E47, transiently in COS7 cells, as interactions between other proteins have been observed in this system (Fig 2-6A). Immunoprecipitations were performed on extracts from transfected cells using a polyclonal antibody against E47 or a monoclonal antibody against the T7 tag. The immunoprecipitates were subjected to SDS-PAGE, and subsequently Western blotted with the anti-T7 or E47 antibodies. Although both proteins were expressed and could be detected by their cognate antibodies, no E47 was precipitated by EBF or vice versa. From these data, we conclude that simple expression of EBF and E47 *in vivo* is insufficient to mediate interaction between these two proteins.

Several different systems were used to test any possible physical interaction between these two proteins. *In vitro* translated ^{35}S labeled proteins used in co-immunoprecipitation experiments did not reveal an interaction between EBF and E47 with or without DNA(data not shown). Polyclonal EBF or E47 antisera were used to immunoprecipitate and Western blot EBF or E47 from B or T cell extracts (Fig. 2-6B). Western blots of anti-EBF immunoprecipitates could detect EBF, but not E47. We think it unlikely that EBF and E47 interact directly, although they may interact indirectly by participating in a higher order protein complex.

Discussion

We have shown that a conserved helix-loop-helix motif in EBF plays a role in transcriptional activation. The function of this domain in B lymphocyte differentiation remains unclear. Recently, an EBF interacting protein was cloned in a one hybrid screen using cDNA from olfactory neurons (Tsai and Reed, 1997). This protein, termed Roaz, contains 29 TFIIIA type zinc fingers. The interaction of Roaz and EBF/Olf-1 was localized to a 253 amino acid domain of EBF that includes the helix-loop-helix domain. Interestingly, Roaz, which binds DNA autonomously, was found to activate or repress transcription by EBF, depending on the promoter context. Roaz is not expressed significantly in lymphoid tissues. It seems possible, even likely, that similar EBF interacting proteins exist to modulate the transcriptional activity of EBF in B cells. The one hybrid approach,

using cDNA from B lymphocytes, may yield better results than the two hybrid screen since no fusion protein containing EBF is required. Due to the complex nature of the structure of the protein, EBF may work best as bait in its native state or with only the transactivation domain deleted, since that construct is known to dimerize and bind DNA well. EBF and its related family members may mediate their activity through distinct interacting proteins in different tissues. Such a mechanism of transcriptional specificity would keep B cell specific genes from being activated in olfactory neurons and vice versa.

EBF itself was originally purified and characterized from fractionated pre-B cell nuclear extracts (Travis et al., 1993). A protein of 115 kD co-purified with EBF through an anion exchange and two DNA affinity columns. Both EBF and the 115 kD protein were gel purified and renatured to test their DNA binding activity. Only EBF bound specifically to the site in the *mb-1* promoter used in the affinity purification, and the 115 kD protein was not studied further. However, the 115 kD protein, while it does not specifically bind the *mb-1* promoter, might be a co-activator that interacts with EBF. Any contribution of the 115 kD protein to transcriptional activation would most likely not be apparent in assays that only test DNA binding. Functional assays testing transcription *in vitro* or *in vivo* may elucidate further the nature of the co-purified protein.

The effect of EBF on transcription in purified *in vitro* systems has not been tested. Another approach to understanding the function of EBF may be to establish a reconstituted *in vitro* transcription system that can support

transcription by EBF. Adding fractionated extracts to a template reconstituted with basal transcription machinery was used successfully to characterize the activity of a CCAAT-box binding protein, CTF (Morgan et al., 1987). The requirements for activation by EBF may become more apparent with this method, which can also incorporate the use of chromatin templates. Experiments discussed in Chapter 3 indicate that EBF may be able to bind an endogenous target site in chromatin. The ability of a factor involved in differentiation, such as EBF, to bind chromatin suggests a role in mediating accessibility to tissue specific genes. This possibility is attractive since locus accessibility of tissue specific genes must be achieved in the early differentiation of any cell type, and thus might account for the conserved function of EBF in many different cell types in diverse species.

Materials & Methods

In vitro Transcription and Translation

Recombinant proteins were generated by coupled *in vitro* transcription /translation using a reticulocyte lysate kit (Promega, TNT). 1 µl out of a 25 µl reaction mix was used for electrophoretic mobility shift assays. Labeled proteins were translated in the presence of 20 µCi ^{35}S methionine and 1 µl of the reaction was separated on a 10% SDS PAGE gel. The gel was dried and subjected to autoradiography.

Protein Extracts and Electrophoretic Mobility Shift Assay

DNA probes were labeled with γ[^{32}P]ATP by incubation with T4 polynucleotide kinase, annealed and then purified on a 5% polyacrylamide TBE gel. One μl *in vitro*-transcribed/translated protein (Promega TNT) was incubated with labeled probe (20,000 cpm, 3 fmol) for 30 min at room temperature in binding buffer (10 mM HEPES pH 7.9; 70 mM KCl; 1 mM DTT; 1 mM EDTA; 2.5 mM $MgCl_2$; 0.05% NP40) with 250 ng sonicated salmon sperm DNA per reaction. DNA competitors and antibodies were added with the DNA probe. The samples were separated on a 6% acrylamide 0.25X TBE gel that was dried and subjected to autoradiography. *(Note: add probe sequence)*

S1 Nuclease Protection Assays

S1 nuclease protection assays were performed essentially as described in Grosschedl and Baltimore, 1985. 5′ [^{32}P] labeled DNA probes for S1 mapping were generated by primer extension of radiolabeled oligonucleotides on a plasmid template. Oligonucleotides used were: (add sequence). The extension product was separated on a 6% denaturating acrylamide gel, purified and hybridized with total RNA at 45° C. The hybrids were digested with 30 units of S1 nuclease at 37° C for 1 hr and separated on a 6% denaturing polyacrylamide-urea gel. Data were quantitated on a PhosphorImager, using ImageQuant software (Molecular Dynamics).

Transfections and immunoprecipitation assays

2 x 10^6 COS7 cells were washed with TS (140 mM NaCl; 5 mM KCl; 25 mM Tris pH 7.4; 0.4 mM NaPhosphate; 50 mM $MgCl_2$; 90 mM $CaCl_2$) and transfected with 5 μg reporter gene construct as indicated together with 0.5 μg RSV-β-galactosidase control plasmid in 0.5 ml TS with 0.5 mg/ml DEAE dextran (Pharmacia) and 0.2 mM chloroquine as described (Grosschedl and Baltimore, 1985). The transfected cells were incubated for 36 hrs and lysed as for nuclear extract preparation. Nuclear extracts from PD36 (pre-B) and EL4 (pro-T) cell lines were prepared according to Schreiber et al., 1989. Protein concentrations were estimated by measuring the absorbance at 280 nm. 100 μg nuclear extracts, precleared with Protein-A Sepharose, were brought up to 0.5 ml in IP Buffer (50mM Tris pH 8.0, 5mM EDTA, 150mM NaCl, 0.5% NP-40). Antibody and 25 μl of 50% Protein-A Sepharose (Pharmacia) was added, and samples were rotated at 4 C for 2 hours to overnight. Samples were spun down and washed four times with SNNTE (50mM Tris pH 8.0, 5mM EDTA, 500mM NaCl, 1% NP-40 and 5% sucrose). Samples were resuspended in SDS sample buffer and run on a 12% SDS-PAGE gel.

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Figure 2-1.

A comparison of the structure and partial sequence of EBF and its Drosophila homolog, collier.

The yellow rectangle represents the zinc coordination and DNA binding domain. Alpha-helical regions are shown in green and blue boxes; the green box corresponds to helix 1 of the HLH motif, while the blue boxes correspond to the duplicated helix 2 (H2 and H2′) of the HLH motif. Deletion analysis has shown that H2 and H2′ are important for homodimerization. The region deleted in EBFΔ296-367 is marked. Amino acid numbers are shown below.

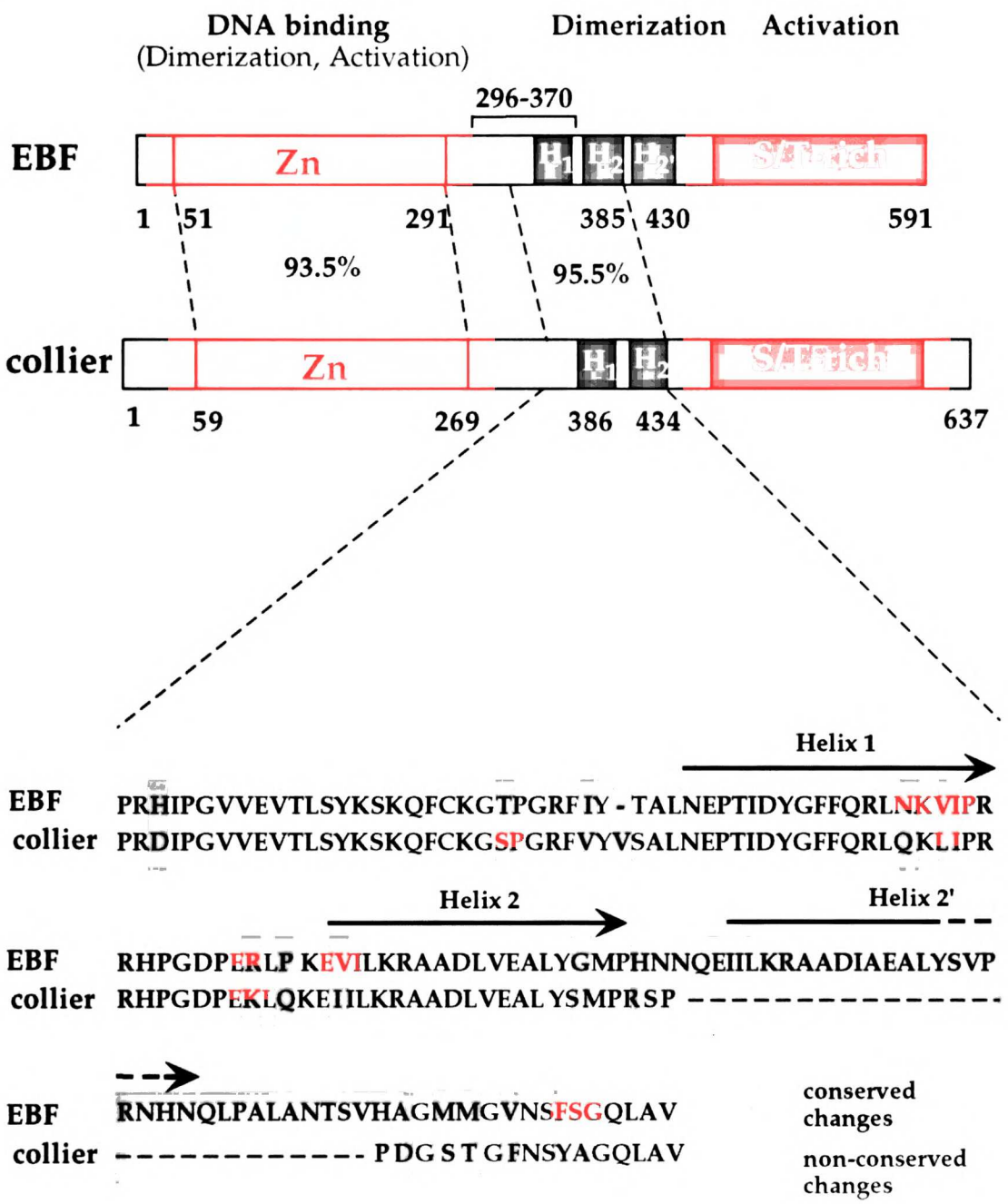

DNA binding
(Dimerization, Activation)
Dimerization
Activation
296-370
EBF
Zn
S/T-rich
1
51
291
385
430
591
93.5%
95.5%
collier
Zn
S/T-rich
1
59
269
386
434
637
Helix 1
EBF PRHIPGVVEVTLSYKSKQFCKGTPGRFIY - TALNEPTIDYGFFQRLNKVIPR
collier PRDIPGVVEVTLSYKSKQFCKGSPGRFVYVSALNEPTIDYGFFQRLQKLIPR
Helix 2
Helix 2'
EBF RHPGDPERLP KEVILKRAADLVEALYGMPHNNQEIILKRAADIAEALYSVP
collier RHPGDPEKLQKEIILKRAADLVEAL YSMPRSP ----------
EBF RNHNQLPALANTSVHAGMMGVNSFSGQLAV
collier ---------- PDG S T G FNSYAGQLAV
conserved changes
non-conserved changes

Figure 2-2.

Collier binds DNA more efficiently than EBF.

(A) SDS-PAGE analysis of *in vitro* transcribed and translated ^{35}S-labeled EBF and collier

(B) An electrophoretic mobility shift assay using equal amounts of EBF and collier is shown. ^{32}P-labeled oligonucleotides containing either an EBF site from the mb-1 promoter (*mb-1*), a consensus EBF site separated by 2 nucleotides (*pal*) or a consensus EBF site separated by 4 nucleotides (*pal+4*) were used as DNA probes. One hundred fold molar excess of unlabeled competitor oligonucleotides was added to the reaction where indicated. The non-specific competitor oligonucleotide, *oct-1*, contains a binding site for the Oct-1 protein. The specific competitor, *mb-1*, is derived from the mb-1 promoter, and contains an EBF binding site.

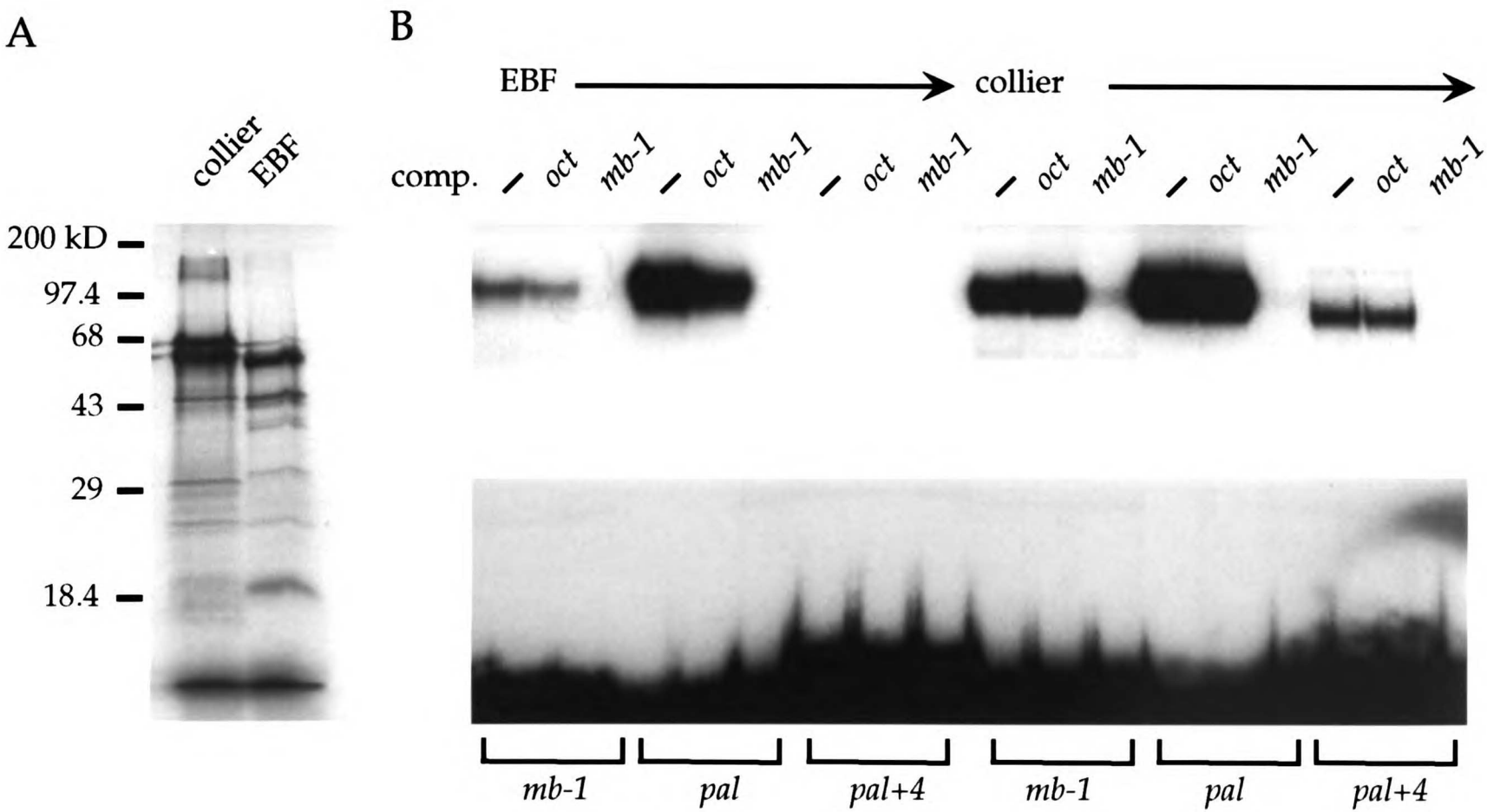

A
collier
EBF
200 kD
97.4
68
43
29
18.4
B
EBF
collier
comp.
oct
mb-1
mb-1
pal
pal+4
mb-1
pal
pal+4

Figure 2-3.

Collier transactivates equally to EBF. Plasmids encoding collier, EBF, an mb-1-OVEC reporter or CMV-OVEC control were transiently transfected in HeLa cells using DEAE-Dextran. The mb-1-OVEC reporter constructs either contained a wildtype EBF site (wt) or a mutant EBF site (mut). RNA was extracted from cell lysates and used in an S1 nuclease protection assay. The migration of the undigested probe, protected fragment, and OVEC control fragment are indicated by black bars.

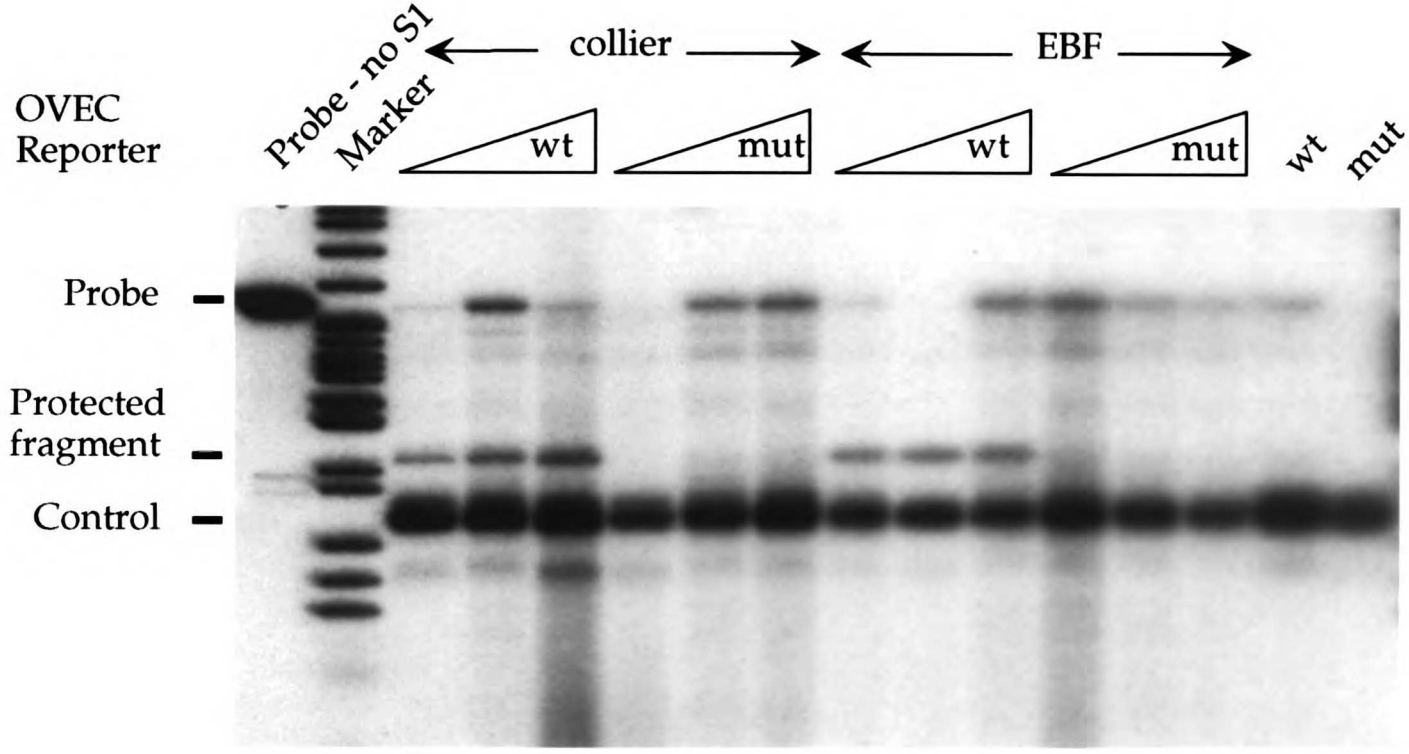

Probe - no S1
Marker
collier
EBF
OVEC
Reporter
wt
mut
wt
mut
wt
mut
Probe
Protected
fragment
Control

Figure 2-4.

A partial deletion of the conserved helix-loop-helix domain of EBF results in decreased transactivation of the mb-1 promoter. Plasmids encoding wildtype EBF, EBFΔ296-367, a wildtype mb-1-OVEC reporter and CMV-OVEC control were transiently transfected into the S194 plasmacytoma cell line. RNA was extracted from cell lysates and used in an S1 nuclease protection assay. Relative transcription units reflect a ratio of reporter expression to control plasmid expression, as quantitated by phosphorimager.

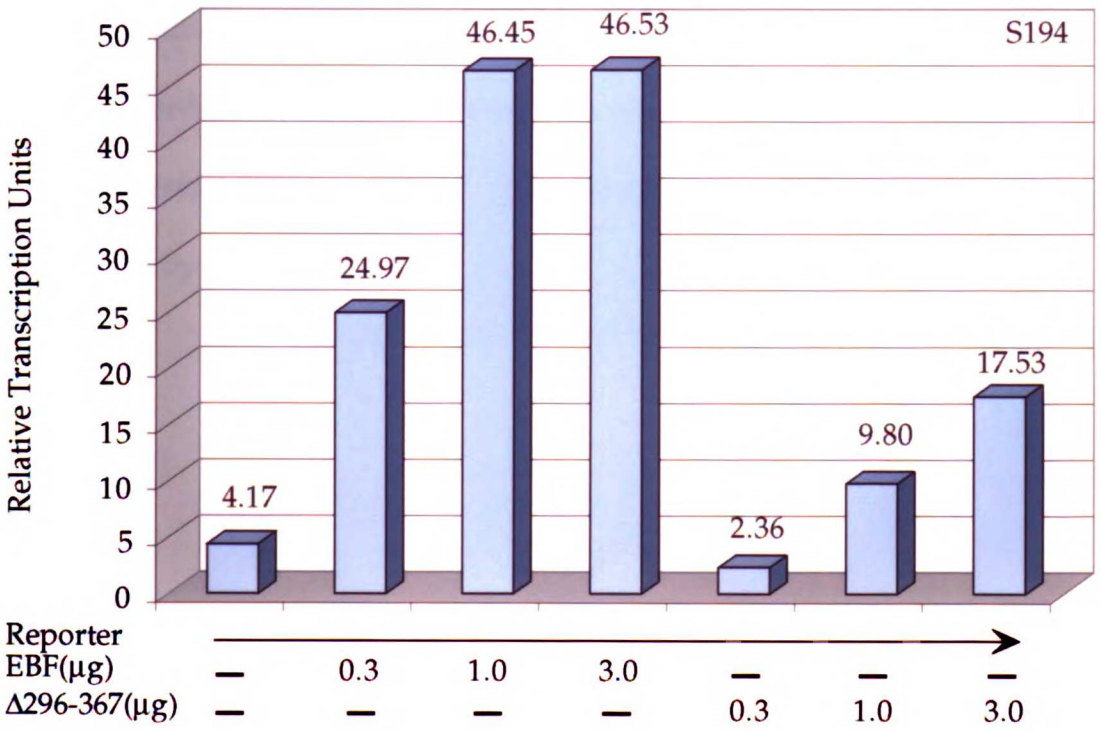
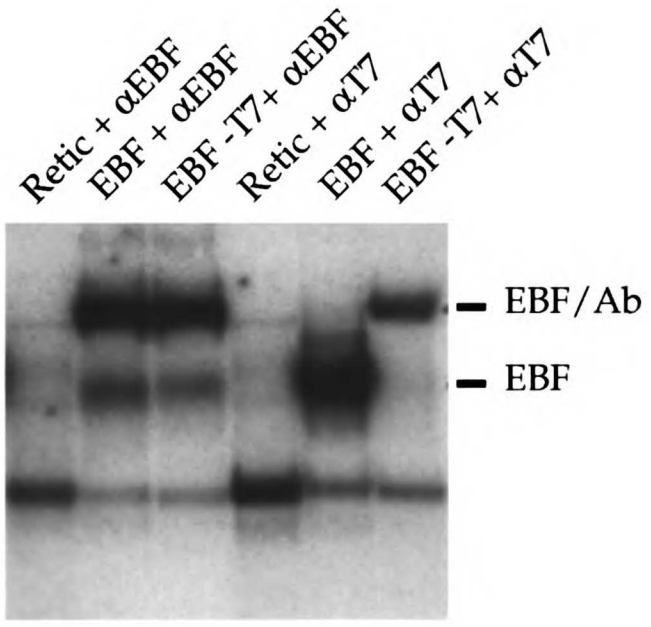

Relative Transcription Units
50
45
40
35
30
25
20
15
10
5
0
4.17
24.97
46.45
46.53
2.36
9.80
17.53
S194
Reporter
EBF(μg)
Δ296-367(μg)
—
0.3
1.0
3.0
—
—
—
—
—
—
0.3
1.0
3.0

Figure 2-5.

A protein of approximately 27 kD co-immunoprecipitates with EBF in COS7 cells.

(A) An electrophoretic mobility shift assay confirming DNA binding and antigenicity of T7-tagged EBF. A ^{32}P labeled oligonucleotide containing an EBF consensus binding site was used as a DNA probe for *in vitro* translated EBF and EBF-T7. Anti-EBF rabbit anti-sera and an anti-T7 monoclonal antibody were used to supershift EBF and EBF-T7. The EBF:DNA and EBF:DNA:Ab complexes are indicated by black bars.

A


Retic + αEBF
EBF + αEBF
EBF -T7+ αEBF
Retic + αT7
EBF + αT7
EBF -T7+ αT7
EBF/Ab
EBF

B

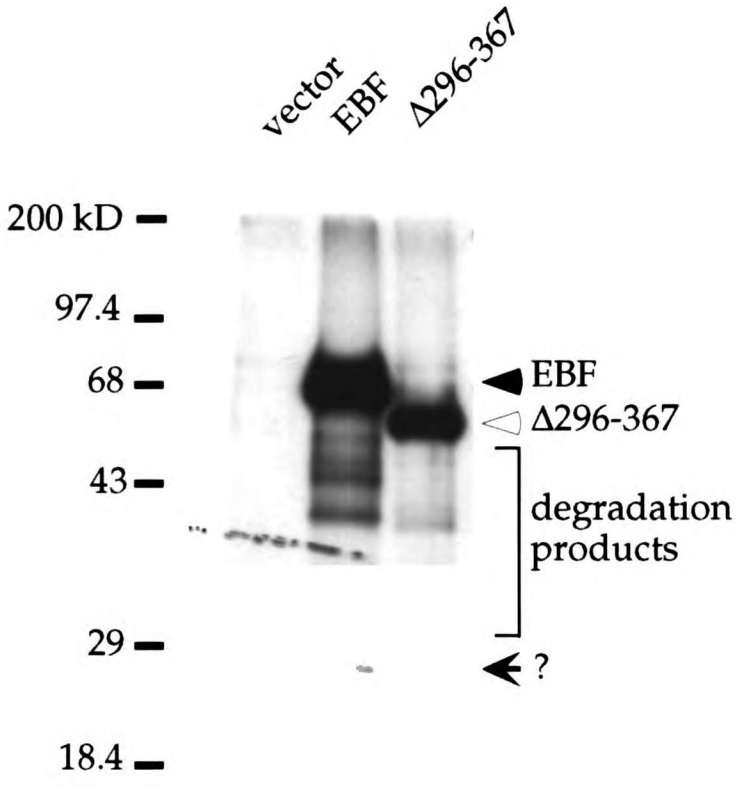
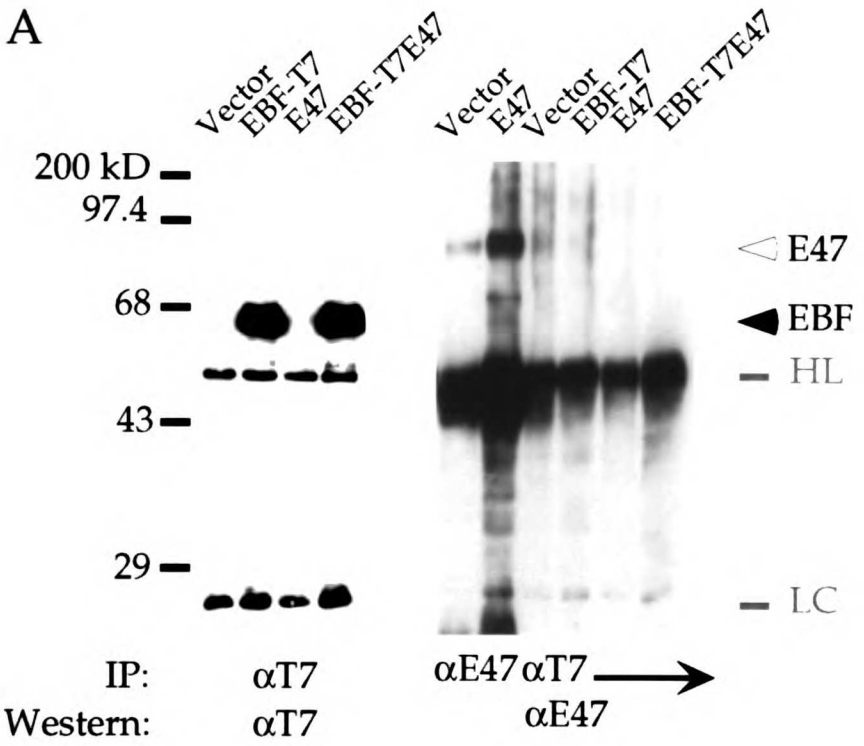

vector
EBF
Δ296-367
200 kD
97.4
68
43
29
18.4
EBF
Δ296-367
degradation products
?

Figure 2-6.

EBF does not directly interact with E47.

(A) Plasmids expressing EBF-T7 or E47 were transfected into COS7 cells by electroporation. Antibodies against the T7 epitope tag (αT7) or E47 (αE47) were added to cell lysates, and precipitated with Protein-A Sepharose. Immunoprecipitates were subjected to Western blotting and probed with antibodies against αT7 or αE47, and conjugated with anti-mouse or anti-rabbit horse radish peroxidase (HRP). Immunoprecipitated native or transfected E47, and EBF are marked by arrowheads. The antibody derived heavy and light chain proteins are marked by black bars.

(B) Native EBF was immunoprecipitated from pre-B or pro-T cell lysates with purified polyclonal anti-EBF antibody. Immunoprecipitates were subjected to SDS-PAGE and Western blotting with αEBF or αE47, or Protein-A Sepharose as a control. Immunoprecipitated EBF is marked by an arrowhead.


A
Vector
EBF-T7
E47
EBF-T7E47
Vector
E47
Vector
EBF-T7
E47
EBF-T7E47
200 kD
97.4
68
43
29
E47
EBF
HL
LC
IP:
αT7
αE47 αT7
Western:
αT7
αE47
B
pro-T
pre-B
pro-T
pre-B
pro-T
pre-B
pro-T
pre-B
97.4 kD
68
43
29
EBF
IP:
pA αEBF
pA αEBF
Western:
αEBF
αE47

CHAPTER 3

EBF and E47 Collaborate to Induce Expression of the Endogenous Ig Surrogate Light Chain Genes

M. Sigvardsson, M. O'Riordan and R. Grosschedl. 1997. EBF and E47 Collaborate to Induce Expression of the Endogenous Immunoglobulin Surrogate Light Chain Genes. *Immunity* 7:25-36.

Abstract

Early B cell factor (EBF) and E47 participate in the transcriptional control of early B lymphocyte differentiation. With the aim of identifying genetic targets for these transcription factors, we stably transfected cDNAs encoding either EBF or a covalent homodimer of E47 into immature hematopoietic BaF/3 cells that lack both factors. In these cells, expression of EBF alone, but not E47, activates low levels of transcription from the endogenous $\lambda 5$ and V_{preB} loci that encode components of the pre-B cell receptor. In combination, EBF and E47 activate the endogenous $\lambda 5$ and V_{preB} loci to levels approaching those observed in pre-B cells, whereas other pre-B cell specific genes remain silent. Multiple functionally important EBF and E47 binding sites were identified in the $\lambda 5$ promoter/enhancer region indicating that $\lambda 5$ is a direct genetic target for EBF and E47. Taken together, these data suggest that the transcription factors EBF and E47 synergize to activate expression of a subset of genes that define an early stage of the B cell lineage.

Introduction

B cell differentiation is a complex developmental process that ultimately generates antibody-secreting cells. This pathway involves multiple stages of differentiation that are defined by the expression of specific cell surface markers and the genomic rearrangement status of the immunoglobulin (Ig) loci. The earliest characterized committed B cell precursor expresses the cell surface markers B220 and AA4.1, and has the Ig heavy chain locus in its germline configuration (Hardy et al., 1991; Li et al., 1996). Subsequent differentiation generates pro-B cells that express the proteins λ5, VpreB, Igα, Igβ, and that rearrange the D and J segments of the Ig heavy chain locus (reviewed in Alt et al., 1987; Melchers et al., 1993). The proteins, λ5 and V_{preB}, serve as surrogate light chains of the pre-B cell receptor which forms after completion of the V to DJ rearrangement of the immunoglobulin μ heavy chain locus in pre-B cells (Karasuyama et al., 1990; Tsubata and Reth, 1990). In mature B cells, the surrogate light chains are replaced with functionally rearranged Igκ or Igλ light chains, generating an IgM antigen receptor, (reviewed in Melchers et al., 1993; Borst et al., 1996). The Igα and Igβ proteins, encoded by the *mb-1* and the *B29* genes (Hombach et al., 1990a,b) respectively, participate in signal transduction through both the pre-B cell receptor and the IgM receptor (Nakamura et al., 1992; Brouns et al., 1993; Papavasiliou et al., 1995). The functional importance of the

pre-B and B cell receptors for cellular differentiation has been demonstrated by targeted inactivation of the genes encoding the various components of these receptors. Mice carrying mutations in the genes encoding the Igμ heavy chain (Ehlich et al., 1993), λ5 (Kitamura et al., 1992) or Igβ (Gong and Nussenzweig, 1996) result in an arrest of B cell differentiation at the pre-B cell stage.

Transcriptional control of B cell differentiation is critically dependent on three genes encoding the transcription factors EBF (Hagman et al., 1991;1993), E47 (Murre et al., 1989a) and BSAP (Barberis et al., 1990). Targeted inactivation of the genes encoding E47 or EBF results in a similar block of B cell differentiation, prior to the rearrangement of the Ig heavy chain locus (Bain et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995). Both EBF- and E47-deficient mice contain, however, some early B cell precursors that express B220 and the IL7 receptor, raising the possibility that neither EBF nor E47 determines the cell fate of the B lymphocyte lineage (Bain et al., 1994; Lin and Grosschedl, 1995). Targeted inactivation of the *Pax5* gene encoding BSAP (B-cell specific activity protein) results in a block of B cell differentiation at a later stage with expression of B cell markers like λ5, VpreB and initiation of D to J recombination in the immunoglobulin heavy chain locus (Urbanek et al., 1994; Nutt et al. 1997).

EBF is a homodimeric transcription factor that is expressed in pro-B, pre-B and B cells, but not in terminally differentiated plasma cells. In addition, EBF is expressed in adipose tissue and in olfactory neurons (Hagman et al., 1993; Wang and Reed, 1993). This transcription factor has been shown to interact with a

functionally important site in the mb-1 promoter (Hagman et al., 1991) and some Ig promoters (Sigvardsson et al., 1996). EBF contains a novel DNA-binding motif that recognizes variants of the palindromic nucleotide sequence 5'ATTCCCNNGGGAAT (Travis et al., 1993). Homodimerization of EBF is mediated by a structural motif that appears to be related to the dimerization motif of helix-loop-helix proteins (Murre et al., 1989b; Hagman et al., 1993).

The E2A gene encodes two distinct proteins, E47 and E12, which are generated by alternative splicing, and are members of the basic helix-loop-helix (bHLH) family of transcription factors (Murre et al., 1989a,b; reviewed in Murre et al., 1994; Kadesch, 1992). E2A proteins bind a consensus E-box sequence 5'CANNTG, which is found in the transcriptional control regions of B cell specific and ubiquitously expressed genes (Ephrussi et al., 1985; Murre et al 1989b; Brennan and Olson, 1990). Both E47 and E12 recognize these sequences as heterodimers with other members of the family of bHLH proteins that are often restricted to specific cell types (Murre et al 1989b; Brennan and Olson 1990; Lassar et al., 1991; Hu et al., 1992). No B cell specific bHLH partners for E47 and E12 have yet been identified (Shen and Kadesch, 1995). Instead, the B cell-specific function of E47 appears to be related to the formation of homodimers that are detected uniquely in B cells (Murre et al., 1991; Bain et al., 1993; Jacobs et al., 1994; Shen and Kadesch, 1995). The formation of an E47 homodimer, identified as the complex, BCF-1, may involve the formation of a covalent

disulfide bond (Benezra, 1995), and is possibly regulated by a B cell specific dephosphorylation of the protein (Sloan et al., 1996).

A specific role for E47 in B cell differentiation was inferred not only from targeted gene inactivation but also from experiments examining the potential of E47 to induce a B cell-like phenotype in non-B cells. Ectopic expression of a E47 cDNA in non-B cells resulted in upregulation of transcription from the genes encoding *TdT*, *Rag1*, *Oct2*, and from the unrearranged μ locus (Schlissel et al., 1991; Choi et al., 1996). None of these putative target genes of E47 can account for the early block in B cell differentiation in mice lacking E47. Mice carrying targeted mutations in the genes encoding *TdT* (Gilfillan et al., 1993; Komori et al., 1993), Rag-1 (Mombaerts et al., 1992), *Rag-2* (Shinkai et al., 1992), *Oct-2* (Corcoran et al., 1993), and *Igμ* (Ehlich et al., 1993) generate pro-B cells that express the surrogate light chain λ5. In contast, mice deficient for the E47 have an earlier block of B differentiation, prior to the expression of λ5 (Bain et al., 1994; Zhuang et al., 1994).

Although the targeted inactivations of EBF and E2A genes demonstrate an essential role of these transcription factors in early B cell differentiation, the absence of pro-B cells in these mice obscures the identification of critical genetic targets. Therefore, we examined whether EBF and E47 homodimers, singly or together, could activate B cell specific genes which might account for the observed phenotype in the deficient mice. Here we show that the stable expression of EBF in the immature hematopoietic cell line, Ba/F3, induces the

expression of the surrogate light chain genes, λ5 and VpreB, but not other lymphoid specific genes such as *Rag1, Il-7R, TdT* and *Pax-5*. In addition, we find that the expression of these genes is markedly increased by co-expression of forced dimers of E47, suggesting that these transcription factors collaborate in the activation of a subset of B cell specific genes.

Results

Stable Ectopic Expression of EBF in Ba/F3 Cells Induces the Expression of λ5 and VpreB

To study the potential role of EBF and E47 in the regulation of B cell differentiation, we chose to express these transcription factors in a non-transformed, immature hematopoietic cell line. Ba/F3 is a bone-marrow derived, IL-3 dependent cell line that may be committed to the B cell differentiation pathway (Palacios and Steinmetz, 1985). However, Ba/F3 cells have been shown to lack the B cell specific homodimer of E47, termed BCF-1 (Murre et al., 1991; Shen and Kadesch, 1995), suggesting that these cells may represent either an early progenitor of B cells or a non-lymphoid cell type. To better define Ba/F3 cells in relation to the B cell lineage, we analyzed the expression of various B cell markers (Figure 3-1). Flow cytometry with antibodies directed against B220 indicated that the Ba/F3 cells lack this pan-B cell marker which was detected on the surface of 70Z/3 pre-B cells (Figure 3-1A). In addition, we examined Ba/F3 cells for the presence of B cell-specific

transcripts by reverse transcriptase-directed polymerase chain reaction (RT-PCR). Ba/F3 cells were found to express *mb-1*, *B29*, germline $I\mu$ transcripts and *E47* (Figure 3-1B). These cells do not contain detectable transcripts from the *Pax-5*, *EBF*, *TdT*, *Rag1*, *VpreB*, *λ5*, *CD19* or *IL-7R* genes, which are expressed in the pre-B cell line 230-238 (Figure 3-1B, and data not shown). As expected, none of the B cell-specific genes examined are expressed in NIH3T3 cells in which E47 transcripts were detected, although at a lower level. We also confirmed the absence of E47 homodimers (BCF-1) and EBF in Ba/F3 cells by analyzing nuclear extracts for the presence of proteins that recognize the E47 and EBF binding sites in an electrophoretic mobility shift assay (Figure 3-1C). Although Oct-binding proteins could be detected in Ba/F3 and 70Z/3 nuclear extracts at similar levels, BCF-1 and EBF were detected only in 70Z/3 nuclear extracts. This phenotype of Ba/F3 cells appeared to be clonally stable and no additional B cell markers were found to be induced by culturing the Ba/F3 in the presence of IL-3 on stromal cells (data not shown). Thus, the Ba/F3 cell line may represent an early B cell precursor that lacks EBF and E47 homodimers, providing a good model system for functional studies of the role of these transcription factors in early B cell differentiation.

To examine the ability of EBF to induce the expression of B cell-specific genes, we stably transfected Ba/F3 cells with a construct encoding a T7-tagged EBF cDNA, together with the neomycin resistance gene. We analyzed 25 neomycin-resistant clones for the expression of EBF by electrophoretic mobility

assays. Five clones were identified that expressed EBF at levels ranging between 3% and 36% of the levels of endogenous EBF in 70Z/3 (Figure 3-2A). Analysis of these EBF-positive Ba/F3 clones for the expression of various B cell markers by a RT-PCR analysis indicated that all five clones contained low levels of transcripts from the V_{preB} and $\lambda 5$ genes but not from the *IL-7R*, *Pax-5* and *Rag-1* genes (Figure 3-2B and data not shown). Neither V_{preB} nor $\lambda 5$ were expressed in the parental Ba/F3 cells or in any of ten neomycin resistant EBF-negative clones (data not shown). Thus, EBF can activate the expression of at least two B cell markers from a silent state.

The $\lambda 5$ Enhancer/Promoter Regions Contains Multiple EBF Binding Sites

The transcriptional activation of V_{preB} and $\lambda 5$ by EBF suggested that these markers may be direct or indirect genetic targets of this transcription factor. The transcriptional control of the $\lambda 5$ gene has been studied in transient transfection assays which have identified a pre-B cell specific enhancer/promoter activity between nucleotides -296 and +65 (Mårtensson and Melchers, 1994; Yang et al., 1995). Inspection of the nucleotide sequence of this transcriptional control region revealed multiple sequence elements that resemble the optimal EBF binding site (Travis et al., 1993) or a consensus E box (Figure 3-3A). Based on the degeneracy of the potential EBF binding sites in the $\lambda 5$ gene, we examined whether oligonucleotides encompassing these sites could interfere with binding of EBF to its site in the mb-1 promoter (Figure 3-3B). Three oligonucleotides from the $\lambda 5$

gene that display sequence similarity with the EBF binding site competed efficiently with DNA binding by recombinant *in vitro* translated EBF. Sites 1 and 3 of the $\lambda 5$ enhancer/promoter region competed as efficiently as the EBF binding site in the mb-1 promoter, whereas site 2 competed with a two-fold lower efficiency. No competition was observed with the unrelated μE5 control oligonucleotide. We further defined EBF binding sites 1 and 2, which deviate from the consensus site, by methylation interference analysis. This analysis identified several specific nucleotides that are contacted by EBF (Figure 3-3C and D).

We confirmed binding of endogenous EBF to site 1 of the $\lambda 5$ enhancer/promoter region by electrophoretic mobility shift assays with nuclear extracts from 230-238 pre-B cells (Figure 3-4A). A single protein:DNA complex was detected which reacted with antiserum directed against EBF but not with antisera directed against the unrelated protein LEF-1.

To study the functional importance of the EBF binding sites in the $\lambda 5$ enhancer/promoter we transiently transfected 230–238 pre-B cells with reporter constructs containing flanking sequences (-299 to +131) from the $\lambda 5$ gene linked to the luciferase coding sequence (Figure 3-4B). Truncation of the $\lambda 5$ enhancer/promoter region to nucleotide -193, which removed two EBF sites and two E-boxes, decreased reporter gene expression to 10% of the level observed with the intact $\lambda 5$ enhancer/promoter region. Transfection of a deletion construct, in which an EBF site and two E-boxes were removed (Δ -141/-58),

resulted in a similar decrease. We also examined directly the role of the EBF-binding sites in the $\lambda 5$ enhancer/promoter by introducing point mutations in site 1, and in both sites 1 and 2. These mutations reduced reporter gene expression to 30% and 7% of the wild type, respectively. Thus, the EBF binding sites are important for the function of the $\lambda 5$ enhancer/promoter region.

The promoter region of the V_{preB} gene also contains potential EBF and E47 binding sites (Kudo and Melchers, 1987). We confirmed by electrophoretic mobility shift assays that both recombinant EBF and E47 can bind to a V_{preB} promoter fragment, but the function of these sites was not examined further (data not shown).

Generation and Expression of an E47 Forced Homodimer

Although EBF induces the expression of $\lambda 5$ and V_{preB} in stably transfected Ba/F3 cells, the levels of expression are relatively low and can be detected only by RT-PCR analysis. In addition, the presence of multiple EBF and E47 binding sites in the $\lambda 5$ promoter and the similarity of the developmental arrest of B cell differentiation in EBF- and E47-deficient mice, raised the question of whether these transcription factors act in concert to regulate a set of genes that is essential for early B cell differentiation.

Functional analysis of E47 and the E47 homodimer, BCF-1, in non-B cells is complicated by the observation that the E47 homodimer is found only in B cells probably due to post-translational modification (Murre et al., 1991; Bain et

al., 1993; Benezra, 1995; Shen and Kadesch, 1995). The regulation of homodimer formation is not well understood. Although transient overexpression of E47 in fibroblasts allows for modest amounts of BCF-1 formation and activation of endogenous genes, the relatively low frequency of gene transfer makes it difficult to quantitate the levels of expression of the induced genes on a per cell basis (Choi et al., 1996). Moreover, the levels of expression of exogenous genes in transiently transfected cells typically exceed those of endogenous genes. Attempts to express E47 in stably transfected Ba/F3 cells did not generate any cell clones expressing high levels of the DNA binding E47 homodimer, BCF-1, (data not shown). This may be due to a lack of regulatory factors required for homodimer formation, however, it is also consistent with previous reports that induced ectopic expression of E47 results in cell cycle arrest of fibroblasts (Peverali et al., 1994). In a stable transfection experiment, clones expressing high levels of E47 may be negatively selected due to cell cycle arrest caused by the heterodimerization of E47 with other regulatory proteins. Therefore, we adopted the approach of generating a homodimeric fusion protein by linking two E47 molecules together in a head-to-tail configuration via a flexible glycine rich linker (Figure 3-5A). This approach has been used successfully to generate functional MyoD-E47 covalent heterodimers in fibroblasts (Neuhold and Wold, 1993). Consistent with the formation of a covalent homodimer, *in vitro*-transcribed and translated E47 forced dimer (E47FD) protein migrates with the predicted molecular mass of approximately 120 kd in a denaturing gel (Figure 3-5B). To

compare DNA binding by E47 and the E47 forced dimer, we examined binding of *in vitro*-translated proteins to a radiolabeled μE5 site in an electrophoretic mobility shift assay (Figure 3-5C). As anticipated, E47 bound DNA with low efficiency whereas abundant DNA binding was detected with the E47 forced dimer. The complex formed between the E47 forced dimer and the μE5 DNA probe could also be supershifted by anti-E47 antibody but not by a control anti-T7 antibody. We confirmed the binding of the E47 forced dimer to the *λ5* enhancer/promoter region by showing that this DNA could compete with the formation of the E47:μE5 protein:DNA complex (Figure 3-5D). In a similar assay, the E47 forced dimer was also able to bind the V_{preB} promoter region (data not shown). In addition, we found that BCF-1 and the E47 forced dimer displayed similar DNA-binding specificity. Neither protein bound to the E-box binding site recognized by the bHLH-Zip protein USF (data not shown) and methylation interference assays showed that both E47 and the E47 forced dimer contact the same E-box DNA residues as those previously reported for E47 (Murre, et al., 1989a). Thus, the covalent linkage of two E47 polypeptides generates a protein that binds DNA with high affinity and with similar specificity as native BCF-1.

EBF and E47 Synergistically Activate the λ5 Enhancer/Promoter Region

To study potential cooperation between EBF and E47 in the activation of the *λ5* promoter, we transiently transfected *λ5* luciferase reporter gene constructs into Ba/F3 and HeLa cells together with expression plasmids encoding EBF or

E47 (Figure 3-6A). Cotransfection of the λ5 luciferase reporter construct with an EBF cDNA resulted in a twenty-fold activation in Ba/F3 cells and a three-fold activation in HeLa cells. In contrast, the E47 expression plasmid did not significantly increase the expression of the reporter gene in either cell type. Cotransfection of the reporter construct with both EBF and E47 expression plasmids markedly increased the activity of the λ5 enhancer/promoter relative to that observed with EBF alone. The E47 forced dimer was functionally comparable to E47 in this transient transfection assay, and in combination with EBF, the forced dimer increased the activity of the λ5 enhancer/promoter up to 240-fold. The transcriptional activation of the reporter gene by EBF and E47 was dependent on the presence of intact EBF binding sites, as shown by the decrease in activation of a reporter construct containing point mutations in all three EBF binding sites of the λ5 enhancer/promoter. A control reporter containing the fos-promoter was not affected by cotransfection with EBF and E47 forced dimers. In these experiments, we noted that the mutations in the three EBF-binding sites did not decrease λ5 enhancer/promoter function to the same low levels as observed in the absence of EBF protein. This discrepancy may be due to the presence of low affinity EBF sites in the λ5 transcriptional control region that can be recognized by EBF in transfected cells that express the protein at high levels. Taken together, these data indicate that EBF and E47 collaborate in the activation of the λ5 enhancer/promoter.

To examine the effect of the EBF site point mutations in the λ5 enhancer/promoter, and to determine whether binding of EBF to the three sites involves cooperativity, we examined the ability of various λ5 enhancer/promoter fragments to compete for binding of EBF to the *mb-1* duplex oligonucleotide (Figure 3-6B). Competition with the wild type enhancer/promoter fragment was only three-fold more efficient than with a fragment containing point mutations in two EBF-binding sites, suggesting that EBF binds to the sites independently of cooperative interactions. As a control, no competition was observed with the enhancer/promoter carrying mutations in all three EBF sites.

We also investigated the contribution of E-boxes to the observed synergy between EBF and E47. Toward this end, we mutated E47 site 1, alone or in combination with EBF site 1, and transiently transfected the mutated λ5 reporter gene construct into 230-238 pre-B cells (Figure 3-6C). Mutation of E47 site 1 decreased the activity of the λ5 promoter two-fold, whereas mutation of EBF site 1 decreased promoter activity four-fold. Mutation of both factor binding sites resulted in a ten-fold decrease in the activity of the λ5 promoter. These data suggest that both EBF and E47 binding sites contribute to the activity of the λ5 promoter, although we did not detect any significant synergy between the two factor binding sites examined. Consistent with this, we failed to observe cooperative DNA binding by EBF and E47 forced dimer to a λ5 DNA fragment containing two E boxes and EBF site 1 (data not shown). Thus, the synergy

between EBF and E47 may depend on the multiplicity of their binding sites in the λ5 enhancer/promoter.

With the aim of examining the potential of EBF and E47/E47 homodimers to cooperatively induce the expression of the endogenous λ5 gene, we transiently transfected Ba/F3 cells with EBF and E47 expression plasmids. Analysis of the transfected cells using an RT-PCR assay indicated that the endogenous λ5 gene was activated by EBF, but not by either E47 or the E47 forced dimer (Figure 3-6D). Co-transfection of EBF and either E47 or the E47 forced dimer did, however, induce higher levels of expression from the λ5 gene, suggesting that EBF and E47 can cooperate to induce expression of the endogenous λ5 gene, as was observed in the stable transfections of Ba/F3 cells. Induction of λ5 expression could also be observed upon transient transfection of EBF and E47 forced dimer expression plasmids into NIH 3T3 fibroblasts, suggesting that activation of the endogenous λ5 gene is not specific for Ba/F3 cells.

Synergistic Transcriptional Activation of the endogenous λ5 and V_{preB} genes by EBF and E47

To study the effects of expressing physiological levels of E47 homodimers, alone or in combination with EBF, we generated stable transfectants in Ba/F3 cells and examined protein expression by electrophoretic mobility shift assays of nuclear extracts (Figure 3-7A and B). We obtained five clones that expressed the

E47 forced dimer alone at levels comparable to the level of endogenous BCF-1 in the pre-B cell line 70Z/3 (Figure 3-7A). However, none of the clones expressed *λ5* or V_{preB} at levels that could be detected by 30 cycles of RT-PCR (data not shown). Moreover, neither *Pax-5, IL7-R, Rag-1* nor *TdT* transcripts were detected in this assay. Previous data suggested that *Iμ* is a target for E47 (Schlissel et al., 1991; Sloan et al., 1996), however, due to the presence of Iμ transcript in the parental Ba/F3 cell line, we were unable to clearly show consistent upregulation of *Iμ* by RT-PCR in the clones that expressed the E47 forced dimers.

We further examined the possibility that EBF and E47 collaborate in the transcriptional activation of B cell specific genes by generating stably transfected Ba/F3 cell lines that express both EBF and an E47 forced dimer (E47FD). In cotransfections of the E47FD gene construct and a puromycin resistant marker into one of the EBF-expressing Ba/F3 cell lines, only 2 out of 45 clones were obtained that expressed both transcription factors, as determined in electrophoretic mobility shift assays (Figure 3-7B). In these experiments, we noted that most E47FD-expressing clones had lost expression of EBF, despite being kept under double selection, implying a selection against cells expressing both proteins. In the two Ba/F3 clones that expressed both EBF and the E47 forced dimer, RT-PCR showed that *λ5* and V_{preB} transcripts were relatively more abundant than in two EBF-positive clones that expressed comparable amounts of EBF (data not shown). To quantitate the levels of target gene expression, we examined these B-cell specific transcripts by S1 nuclease protection assays of total

RNA (Figure 3-7C). In Ba/F3 cell clones expressing either EBF or the E47 forced dimer alone, no $\lambda 5$ or V_{preB} transcripts were found, whereas in the two cell clones expressing both transcription factors, $\lambda 5$ transcripts were readily detected. The levels of expression were calculated at 84% and 17% of the levels detected in the pre-B cell line 230-238, when normalized to the levels of the β-actin control transcript. The clones expressing both EBF and E47FD, were also found to contain V_{preB} RNA at 19% and 3% of the level detected in 230-238 cells. Thus, EBF and the E47 forced dimer synergize in activating expression of the endogenous $\lambda 5$ and V_{preB} loci from a transcriptionally silent state in Ba/F3 cells.

Discussion

The transcription factors EBF and E47 have been shown to regulate early stages of B cell differentiation (Bain et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995). The pronounced similarity of the phenotype of EBF- and E47-deficient mice raised the possibility that these transcription factors regulate a similar set of genes. However, the complete block of B cell differentiation in both mutant strains of mice has complicated the identification of important target genes. Here we show that the transcription control regions of both the $\lambda 5$ and V_{preB} genes contain multiple EBF- and E47-binding sites, indicating that these genes are direct targets of EBF and E47. We also find that the $\lambda 5$ enhancer/promoter region can be activated strongly by EBF and E47 in transiently transfected HeLa cells in a site dependent manner. Finally, stable

expression of EBF and E47 homodimers in immature hematopoietic Ba/F3 cells induces transcription of the genes encoding the Ig surrogate light chains, λ5 and V_{preB}. Notably, EBF and E47 act in concert to induce the expression of the endogenous *λ5* and *V_{preB}* genes from a transcriptionally silent state to levels approaching those found in pre-B cell lines. Induction of endogenous *λ5* gene expression by EBF and E47 can also be observed in transiently transfected Ba/F3 and NIH 3T3 fibroblastic cells.

Cell type-specific expression of genes is governed by transcription factors that are restricted in their cell type distribution. In yeast, a simple paradigm for cellular differentiation, a single transcription factor is sufficient to induce a specific cellular phenotype. Ectopic expression of the α1 gene in cells of the a mating type induces an entire set of α specific genes (reviewed in Herskowitz, 1989). In higher eukaryotes, a set of myogenic bHLH proteins, MyoD, myogenin, Myf5 and MRF4 can each induce morphological changes and transcriptional activation of muscle-specific genes when introduced into non-muscle cell types (reviewed in Olson, 1990; Weintraub, 1991; Emerson, 1993). The efficiency of MyoD-induced myogenesis is typically low, but can be significantly enhanced by co-expression of any of these bHLH proteins with the transcription factor MEF2 (Molkentin et al., 1995). A similar cooperation between two cell type-specific transcription factors C/EBPα and PPARγ2 is required for the efficient induction of adipogenesis in fibroblastic cell lines (Tontonoz et al., 1994). The synergy of EBF and E47 homodimers in activating the pro B cell markers □5 and VpreB

resembles the cooperation of cell type-transcription factors in myogenesis and adipogenesis. In contrast to those differentiation systems, the collaboration of EBF and E47 results in the induction of only a small subset of lineage specific markers rather than an entire differentiation program. This difference may reflect the multistep process of B cell differentiation (reviewed in Alt et al., 1987; Melchers et al., 1993) or the inability of EBF and E47 homodimers to function as lineage commitment factors.

Various mechanisms have been shown to contribute to synergy between transcription factors. First, cooperative DNA binding by two transcription factors can increase both the affinity of DNA binding and the diversity of the transcriptional response. For example, the yeast α2 repressor protein, which alone binds DNA inefficiently, can be recruited to its site by cooperative binding with MCM protein which by itself functions as an activator of transcription (Keleher et al., 1988). Second, collaboration between distinct transcription factors may also involve protein:protein interactions independent of changes in DNA binding. The transcriptional activation potential of MyoD:E12 heterodimers can be greatly augmented by their association with the DNA-binding domain of MEF2. Although EBF and E47 contain dimerization motifs that share sequence similarities and both proteins display a strong functional synergy in activating pre-B cell specific genes, we have been unable to co-immunoprecipitate *in vitro* translated EBF and E47 (data not shown). Moreover, we find that co-incubation of EBF and E47 homodimers with a probe containing both binding sites yields

predominantly single factor/DNA complexes. Thus, the synergy between EBF and E47 in the regulation of the $\lambda 5$ gene may be independent of direct protein:protein interactions. Instead, the multiplicity of binding sites for EBF and E47 may account for the magnitude of the collaborative activation of gene expression. It is also possible that multiple binding sites for regulatory factors such as EBF and BCF-1, may facilitate the activation of target genes in the context of nuclear chromatin.

EBF and E47 homodimers appear to differ in their potential to activate transcription of the $\lambda 5$ and V_{preB} genes. EBF, but not the E47 forced dimer, is able to induce low levels of expression of the endogenous $\lambda 5$ and V_{preB} genes in Ba/F3 cells. This observation raises the possibility that EBF may be the primary determinant in the activation of the silent $\lambda 5$ and V_{preB} loci, and that E47 collaborates with EBF to significantly increase the level of transcription. E47 has been shown previously to act as a potent transcriptional activator (Quong et al., 1993), and consistent with this, the E47 forced dimer is capable of activating the $\lambda 5$ promoter in transient transfection assays (Figure 6A). In addition, the relative levels of transcription of the $\lambda 5$ and V_{preB} genes in the two Ba/F3 cell clones which express both EBF and the E47 forced dimer correlates with the abundance of the E47 forced dimer and not with that of EBF. Thus, EBF may be capable of acting on regulatory regions in the context of transcriptionally inactive chromatin, whereas E47 may provide strong transcriptional activation functions.

In addition to EBF and E47, other B cell-specific transcription factors may contribute to the regulation of the $\lambda 5$ and V_{preB} genes. For example, both the $\lambda 5$ and V_{preB} enhancers contain binding sites for Pax5 (BSAP, EBB1) (Yang et al., 1995; Okabe et al., 1992b) and the absence of *Pax5* transcripts in Ba/F3 cells could account for the lower levels of $\lambda 5$ and V_{preB} gene expression as compared to pre-B cells. It is also important to note that the expression patterns of EBF, $\lambda 5$ and V_{preB} are overlapping but not identical, either in tissue distribution (Hagman et al., 1993; Wang and Reed, 1993), or during B cell differentiation (Kudo and Melchers, 1987; Kudo et al., 1987; Okabe et al., 1992a; Hagman et al., 1991; Hagman et al., 1993). The more restricted distribution of $\lambda 5$ and V_{preB} may be explained by the presence of repressor elements in the control regions of these genes (Mårtensson and Melchers, 1994; Okabe et al., 1992a) which may be important in both spatial and temporal regulation.

The E47 protein has been previously shown to regulate the progression of the cell cycle (Peverali et al., 1994). Experiments in which the levels of E47 protein were increased either with an inducible expression system in stably transfected NIH 3T3 cells, or with microinjection of antibodies directed against endogenous Id, indicated that E47 has a cell growth-suppressive activity (Peverali et al., 1994). The HLH protein, Id, forms a heterodimeric complex with E47 that is unable to bind DNA (Benezra et al., 1990) and overexpression of Id1 in transgenic mice results in a block of B cell differentiation with a markedly reduced level of $\lambda 5$ transcripts (Sun, 1994). In our stable transfection

experiments with the E47 forced dimer, we have not observed any significant change in the proliferation of cells. This may be due to our choice of an immature hematopoietic cell line instead of fibroblast cells for our transfections or, alternatively, that the cell cycle block is a result of a heterodimeric complex of E47 with another protein.

Previous experiments in which E47 cDNA had been transiently or stably transfected into fibroblasts or pre T cells identified the *Rag1*, *Iμ* and *TdT* genes as targets for E47 (Schlissel et al., 1991; Choi et al., 1996). We did not detect significant levels of *TdT* or *Rag1* transcripts in Ba/F3 cells stably transfected with either the E47 forced dimer alone or with the E47 forced dimer in combination with EBF, nor was *Iμ* upregulated consistently. The ectopic activation of the *Rag1*, *TdT* and *Iμ* genes, which are lymphoid specific rather than B cell specific, may be mediated by E47 in complex with another protein.

Our data strongly suggest that one function of the transcription factors EBF and E47 is to activate a subset of early B cell specific genes. Although our data identify the *λ5* and V_{preB} genes as genetic targets for EBF and E47, the targeted inactivation of the *λ5* gene results in a phenotype distinct from the early block of B cell differentiation in EBF- and E47-deficient mice (Kitamura et al., 1992; Bain et al., 1994; Zhuang et al., 1994). A targeted inactivation of *λ5* and both functionally equivalent murine V_{preB} genes (Kudo and Melchers, 1987) may be needed to address the question of whether the combined absence of these proteins can account for the early block of B cell differentiation in the EBF- or

E47- deficient mice. It is also possible that other genes are regulated by EBF and E47. We have considered *mb-1* to be a candidate target gene for EBF due to the presence of a functionally important site in the promoter which binds EBF (Hagman et al., 1991). Furthermore, EBF and mb-1 share a similar expression pattern in the B cell lineage (Travis et al., 1991; Hagman et al., 1991; 1993). However, the *mb-1* gene is expressed in Ba/F3 cells in the absence of EBF and BCF-1, and of BSAP, which binds the *mb-1* promoter in cooperation with an Ets family protein (Fitzsimmons, et al., 1996). Thus, only a subset of cell type specific genes that contain binding sites for a particular transcription factor may represent *in vivo* genetic targets. Further experiments will be required to define any additional functions of EBF and E47 during B-cell development and maturation.

Materials & Methods

Cell Lines and Flow Cytometry

Lymphoid cells (70Z/3 and 230-238) were maintained in RPMI (GIBCO) supplemented with 50 μM β-mercaptoethanol, 1 mg/ml Penicillin-Streptomycin and 10% fetal calf serum. For the maintenance of Ba/F3 cells the culture medium was supplemented with 10% conditioned medium from WEHI3 cells as a source of IL-3. HeLa cells were grown in DMEM (GIBCO) supplemented with 1 mg/ml Penicillin-Streptomycin and 5% fetal calf serum.

For flow cytometry, 10^6 cells were washed with PBS containing 3% BSA and incubated 30 min with phycoerythrin-conjugated rat anti-mouse B220 antibody (Caltag). The cells were washed twice in PBS/BSA and the samples were analyzed in a FACScalibur flow cytometer using CellQuest software (Becton Dickinson).

Gene Constructs and Site-Directed Mutagenesis

The EBF cDNA used in all the experiments was modified at the C terminus with a T7 tag sequence by PCR, using the primers: EBF-PVU2 5'CTTCCTCAATGGCTCAGCTGC3'; T7CTag/EBF 5'GATCTAGACTAGCCCA TCTGCTGGCCGCCGGTCATGCTCATGGG AGGGACAATCATGC. Stable cell lines were made with EBF-T7 in pcDNA3 (Invitrogen). The EBF-T7 cDNA3 construct contains 45 nucleotides of the 5'UTR. The E47 forced homodimer (E47FD) construct was generated by joining two copies of a syrian hamster E47 cDNA, E47mutC and E47mutN, with a linker encoding multiple glycines and serines. E47mutN incorporates an Nde I site at the N-terminal ATG of E47. E47mutC contains an Xho I site replacing the C-terminal stop codon. The reconstruction sites were introduced by site-directed mutagenesis: E47mutC 5'CCGGGCACCTCGAGCTGCCACATGG; E47mutN 5' GCCCTGGCCATA TGATGAACC. E47mutC was digested with Xho I and ligated to the peptide linker which contains an N-terminal Xho I site and a C-terminal Nde I site (peptide linker 5'TCGAGGGGACAGGGGGGGGAGCGGGGGGGGAGCGG

GGGGGGGAGCGGGGGGGGGAGCGGGGGGGGGACACA) to produce the construct E47mutC/linker and E47mutN, which were then digested with Nde I and Xba I. The appropriate digestion fragments were ligated together to produce the E47FD construct in pcDNA3 (Invitrogen).

The λ5 enhancer was cloned by PCR amplification from genomic DNA using primers amplifying a 430 bp fragment containing sequences from nucleotide -299 to the translation start site at nucleotide +131. The primers (sense: 5'GGGGTACCAGAGACTCTTGTTCCATGG; antisense: 5'CCGCTCGAG TCTAGCCTCACTTGCAG) included restriction sites to facilitate the cloning of the enhancer into the KpnI and XhoI sites of the luciferase reporter vector, pGL3 basic (Promega). Point mutations were introduced by combinations of PCR and restriction enzyme digestions to generate the mutant enhancers. Primers for PCR mutagenesis were used in combination with the λ5 primers described above: mEBF site 1 antisense 5'CCATGGTCACCATCTGTGGAGTTCTCTAT TAAATTACTACCCGGTTGTG; mEBF site 2 sense: 5'CCAGGGGCCCTAATA TACTGGATATCAGTCAGGC; mEBF site 3 antisense 5'TATTAGGGCCCC TGGGTCTGTGGAGCAGGTAGCTACTGCTTAGAGGGGCC; mE-box 1 anti-sense 5'CCATGGTCACCTTCCGTGGAGTTCTCT.

Stable Cell Transfections

Ba/F3 cells were transfected with the expression vector pcDNA3 containing EBF-T7 or E47FD cDNA under the control of the cytomegalovirus

(CMV) promoter. 10^7 cells were electroporated (960 μF, 250V) with 2 μg of plasmid in 0.5 ml medium. After 24 hr, the cells were diluted in 150 ml medium containing 0.75 mg/ml active Neomycin (Geneticin, GIBCO). The cells were plated into 24-well plates and incubated until colonies were visible (10–14 days). Cells from wells containing single colonies were expanded and nuclear extracts were prepared as described by Schreiber et al. (1989). Double transfected Ba/F3 cell lines were generated by sequential transfections of 2 μg EBF cDNA plasmid containing a linked neomycin resistance gene, neoR, and 10 μg E47FD cDNA construct together with 1 μg puromycin resistance gene. Neomycin and puromycin resistant cell clones were screened for the presence of both the E47FD and EBF activity in electrophoretic mobility shift assays (see below).

Transient Transfections and Luciferase Assays

2 x 10^6 230–238 pre-B cells, Ba/F3 cells or Hela cells were washed with TS (140 mM NaCl; 5 mM KCl; 25 mM Tris pH 7.4; 0.4 mM NaPhosphate; 50 mM $MgCl_2$; 90 mM $CaCl_2$) and transfected with 0.5-2 μg reporter gene construct as indicated together with 0.5 μg RSV-β-galactosidase control plasmid in 0.5 ml TS with 0.5 mg/ml DEAE dextran (Pharmacia) and 0.2 mM chloroquine as described (Grosschedl and Baltimore, 1985). The transfected cells were incubated for 36 hrs.

Preparation of protein extracts and luciferase assays were performed with a luciferase assay kit (Promega) using 10% of the protein extract. β-galactosidase

assays were performed with 10% of the same protein extract in 40 µl reaction buffer (150 mM Tris pH 7.5; 10 mM $MgCl_2$; 25 mM NaCl; 10 mM β-mercaptoethanol; 1 mg/ml BSA; 0.15 mM 4-methylumbelliferyl β-galactosidase) for 30 min at room temperature. The reactions were stopped by the addition of 1 ml 0.25 M glycine pH 10.65 and the conversion of substrate was measured in a Hoefer DNA fluorometer TKO 100.

Transient transfections to study the induction of the endogenous *λ5* gene, were done by electroporation (see above) of 5 x 10^6 cells with 15 µg of expression plasmid. The cells were harvested after 48 hours and RT-PCR analysis was performed as described below.

Protein Extracts and Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to Schreiber et al., 1989. Protein concentrations were estimated by measuring the absorbance at 280 nm. DNA probes were labeled with γ[^{32}P]ATP by incubation with T4 polynucleotide kinase, annealed and then purified on a 5% polyacrylamide TBE gel. Five µg nuclear extract or 1 µl *in vitro*-transcribed/translated protein (Promega TNT) was incubated with labeled probe (20,000 cpm, 3 fmol) for 30 min at room temperature in binding buffer (10 mM HEPES pH 7.9; 70 mM KCl; 1 mM DTT; 1 mM EDTA; 2.5 mM $MgCl_2$; 0.05% NP40) with 0.75 µg Poly dI/dC (Pharmacia). DNA competitors and antibodies were added 10 min before the addition of the DNA probe. The samples were separated on a 6% acrylamide TBE gel which

was dried and subjected to autoradiography. Data were quantitated on a PhosphorImager, using ImageQuant software (Molecular Dynamics).

Oligonucleotides used for electrophoretic mobility shift assays were: Oct sense 5'GATCTGCTTCTTAATAATTTGCATACCCTCACTG; Oct antisense 5'GA TCCAGTGAGGGTATGCAAATTATTAAGAAGCA; μE5 sense 5'GGCCAGAA CACCTGCAGACG; μE5 antisense 5'CGTCTGCAGGTGTTCTGGCC; mb-1 EBF sense 5'GAGAGAGACTCAAGGGAATTGTGG; mb-1 EBF antisense 5'CCAC AATTCCCTTGAGTCTCTCTC; λ5 EBF 1 sense 5'TCGACAACCGGGTAGTC CTTTGGGAGA-GAACTCCACAGATGG; λ5 EBF 1 antisense 5'TCGACCATC TGTGGAGTTCTCTCCCAAAGGACTACCCGGTTG; λ5 EBF 2 sense 5'TCGAC CCAGGGGCCCTCAGGGACTGGATATCAG; λ5 EBF 2 antisense 5'TCGACTG ATATCCAGTCCCTGAGGGCCCCTGGG; λ5 EBF 3 sense 5'CCCCTCTAAGCC CTGGGGACCTGCTCCACAGAC; λ5 EBF 3 antisense 5'GTCTGTGGAGCAGGT CCCCAGGGCTTAGAGGGG.

Reverse Transcriptase and Polymerase Chain Reactions

RNA was prepared from cells using an RNA extraction kit (Biotecx Inc.) cDNA was generated by annealing 1 μg total RNA and 1 pmol of random hexamers (Pharmacia) in 10 μl DEPC-treated water. Reverse transcriptase reactions were performed with 2 units MMLV-RT (Boehringer) in the manufacturers' buffer supplemented with 1 mM dNTP and 0.05 units/μl RNAsin (Promega) in a total

volume of 20 μl, at 37 °C for 1 hr. One-twentieth of the RT reaction was used for the PCR assays.

PCR reactions were performed with 1 unit Taq-polymerase (Boehringer Mannheim) in the manufacturers' buffer supplemented with 0.2 mM dNTP, in a total volume of 10 μl. The condition of each PCR cycle was (unless indicated otherwise) 94°C, 30 sec; 55°C, 1 min; 72°C 45 sec. Primers were added to a final concentration of 1 mM. 22 cycles were used for Actin RT-PCR, 30 cycles for all other primer sets. Primers used: Actin sense 5'GTTTGAGACCTTCAACACC; Actin antisense 5'GTGGCCATCTCCTGCTCGAAGTC; B29 sense 5'GGTGAGC CGGTACCAGCAATG; B29 antisense 5'AGTTCCGTGCCACAGCTGTCG; V_{preB} sense 5'CGTCTGTCCTGCTCATGCT; V_{preB} antisense 5'ACGGCACAGTAA TACACAGCC; $\lambda 5$ sense 5'TGTGAAGTTCTCCTCCTGCTG; $\lambda 5$ antisense 5'ACCACCAAAGTACCTGGGTAG; mb-1 sense 5'GCCAGGGGGTCTA GAAGC; mb-1 antisense 5'TCACTTGGCACCCAGTACAA; Iμ sense 5'ACCTGGGAATGTATGGTTGTGGCTT; Iμ antisense 5'ATGCAGATCTCTGTT-TTTGCCTCC; Rag1 sense 5'TGCAGACA TTCTAGCACTCTGG; Rag1 antisense 5'ACATCTGCCTTCACGTCGAT; TdT sense 5'GAAGATGGGAACA ACTCGAAGAG; TdT antisense 5'TGGCAGAGATTTCAGTACAGAGG; Pax5 sense 5'CTACAGGCTCCGTGACGCAG; Pax5 antisense 5'GTCTCGGCCTGTG AAATAGG; E47 sense 5'GACGCCGAAGAGGACAAGAA; E47 antisense 5'CAGGATCACCGTCACCGCCT.

In vitro Transcription and Translation

Recombinant proteins were generated by coupled *in vitro* transcription/translation using a reticulocyte lysate kit (Promega, TNT). 1 µl out of a 25 □l reaction mix was used for electrophoretic mobility shift assays. Labeled proteins were translated in the presence of 20 µCi ^{35}S methionine and 1 µl of the reaction was separated on a 10% SDS PAGE gel. The gel was dried and subjected to autoradiography.

Methylation Interference and S1 Nuclease Protection Assays

Methylation of 5'[^{32}P] labeled oligonucleotides was performed as described (Hagman et al., 1991). 10^6 cpm of probe DNA in 10 µl TE was methylated by adding 1 µl di-methyl sulfate (DMS) in DMS buffer (5mM sodium cacodylate, pH 8.0; 1mM EDTA pH 8.0) for five minutes at room temperature. Forty µl of stop buffer (1.5M sodium acetate, pH 7.0, 1M β-mercaptoethanol) was added with 10 µg tRNA. The probe was ethanol precipitated three times. The methylated DNA was incubated with *in vitro*-transcribed/translated EBF and the bands representing bound and free DNA were excised, extracted in TE, ethanol precipitated, boiled in 1M piperidine for 25 min, lyophilized three times, resuspended in sequence loading buffer and separated on a 10% sequencing gel.

S1 nuclease protection assays were performed essentially as described in Grosschedl and Baltimore, 1985. 5' [^{32}P] labeled DNA probes for S1 mapping were generated by primer extension of radiolabeled oligonucleotides on a

plasmid template. Oligonucleotides used were: actin 5'GGCCATCTCCTGCTCGAAGTC; $\lambda 5$ 5'CCTACTCTGAGCTTCATTGACC; V_{preB} 5'GGACAGACGTCCAGGCCATG. The extension product was separated on a 6% denaturating acrylamide gel, purified and hybridized with total RNA at 45° C. The hybrids were digested with 30 units of S1 nuclease at 37° C for 1 hr and separated on a 6% denaturing polyacrylamide-urea gel. Data were quantitated on a PhosphorImager, using ImageQuant software (Molecular Dynamics).

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Figure Legends

Figure 3-1.

Ba/F3 cells display a subset of pre-pro B cell markers.

(A) Ba/F3 cells do not express the surface antigen B220. Flow cytometry analysis of expression of the B cell lineage marker B220 on the surface of the pre-B cell line, 70Z/3, and on cells of the immature hematopoietic cell line Ba/F3. The filled area indicates the background PE signal without antibody and the open area, the PE signal obtained after antibody addition.

(B) Ba/F3 cells express *Iμ*, *B29* and *mb-1* transcripts but not other early B cell markers. Ethidium bromide-stained agarose gels showing the products of a qualitative RT-PCR analysis from 30 cycles of total RNA from Ba/F3 cells, 230-238 pre-B cells and NIH 3T3 fibroblasts.

(C) Ba/F3 cells lack the B cell specific DNA-binding proteins, EBF and BCF-1. Electrophoretic mobility shift assays of nuclear extracts (5 μg) from Ba/F3 cells, 70Z/3 pre-B cells, or HeLa cells. The nuclear extracts were incubated with either [^{32}P] labeled octamer (Oct probe), μE5 (E47 probe) or mb-1 (EBF probe) oligonucleotides. The positions of the Oct:DNA, EBF:DNA and BCF-1 (E47 homodimer) DNA complexes are indicated by arrowheads.

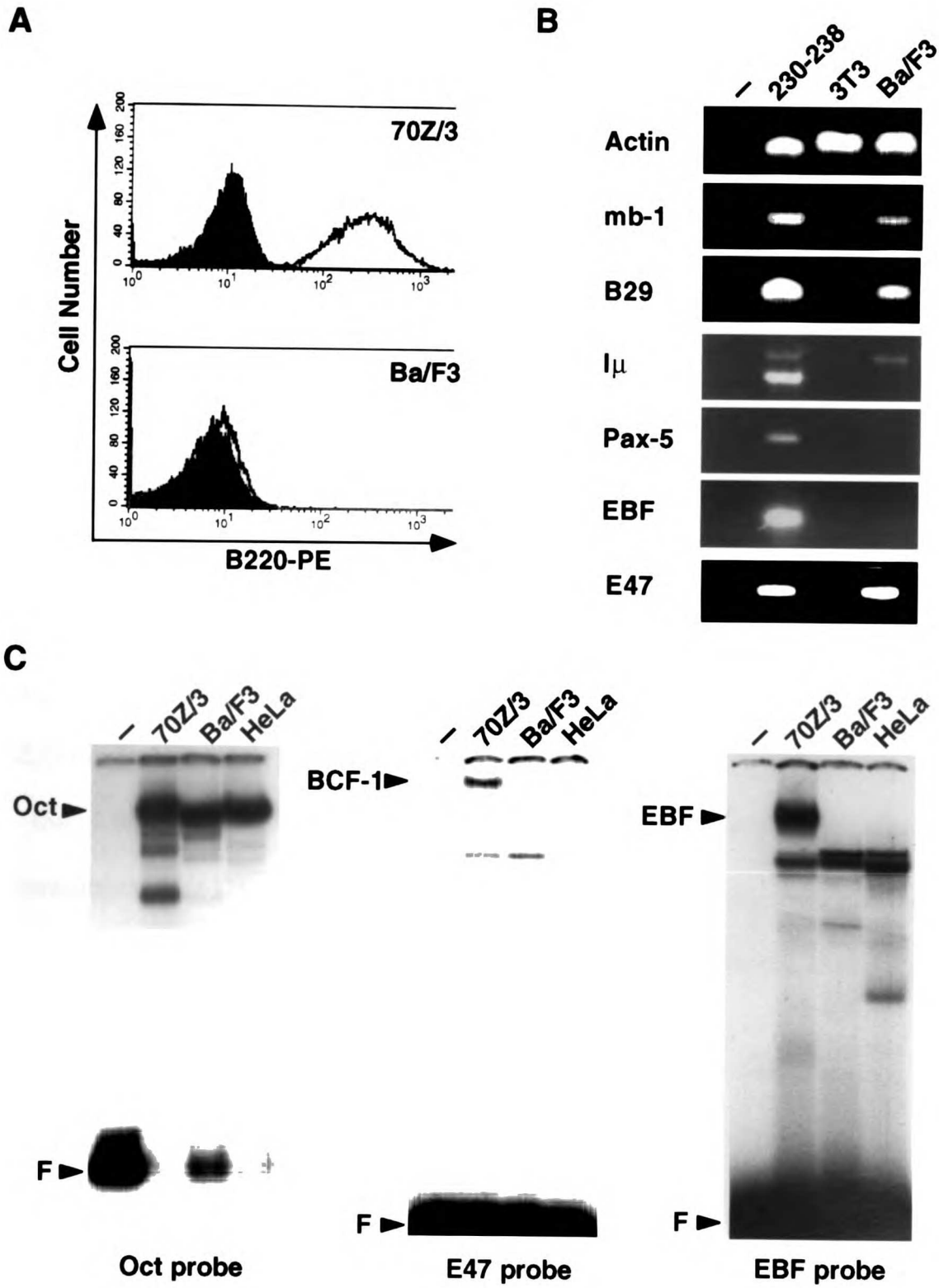

A
70Z/3
Ba/F3
Cell Number
B220-PE
B
–
230-238
3T3
Ba/F3
Actin
mb-1
B29
Iμ
Pax-5
EBF
E47
C
–
70Z/3
Ba/F3
HeLa
Oct
F
Oct probe
BCF-1
E47 probe
EBF
EBF probe

Figure 3-2.

Expression of EBF in Ba/F3 cells induces transcriptional activation of the $\lambda 5$ and V_{preB} genes at low levels.

(A) Electrophoretic mobility shift assay to detect EBF expression in Ba/F3 cells stably transfected with an EBF cDNA. Nuclear extract (5 μg) from each cell clone was incubated with an EBF-binding site from the mb-1 promoter. Nuclear extracts from 70Z/3 pre-B cells and Hela cells were used as positive and negative controls, respectively. The protein:DNA complexes were separated on a 6% polyacrylamide gel. The region of the EBF:DNA complex in the autoradiogram is shown, and the relative amounts of the complex were quantitated by Phosphor Imager analysis.

(B) $\lambda 5$ and V_{preB} expression in EBF-positive Ba/F3 cells. Qualitative RT-PCR analysis of total RNA from parental and transfected Ba/F3 clones, and from 70Z/3 pre-B cells. The top panel shows an ethidium bromide-stained agarose gel of the β-actin RT-PCR analysis (20 cycles). The lower panels display autoradiograms of DNA blots of the $\lambda 5$ and V_{preB} RT-PCR amplification products (30 cycles). The cDNA from 70Z/3 cells was diluted five-fold for the $\lambda 5$ and V_{preB} RT-PCR analysis.

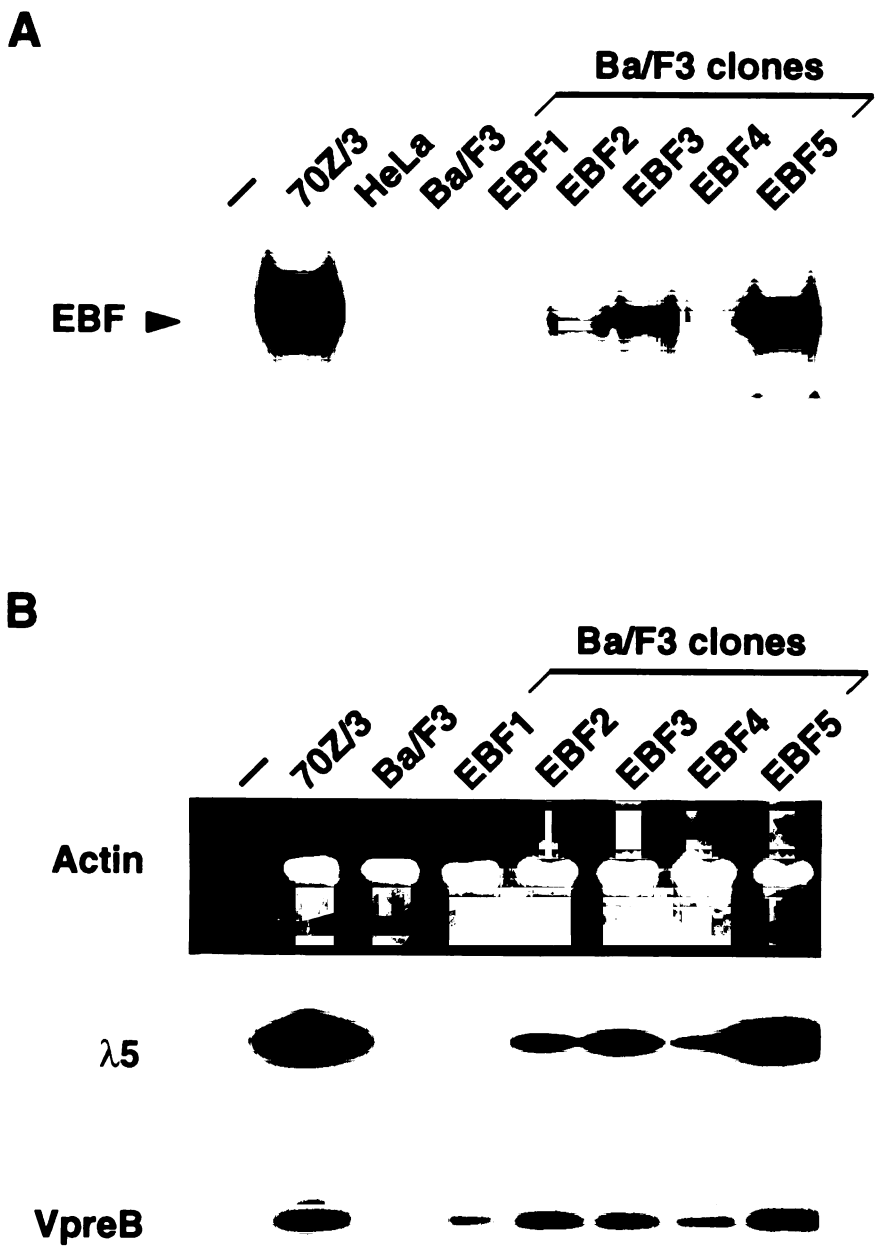

A
Ba/F3 clones
—
70Z/3
HeLa
Ba/F3
EBF1
EBF2
EBF3
EBF4
EBF5
EBF
B
Ba/F3 clones
—
70Z/3
Ba/F3
EBF1
EBF2
EBF3
EBF4
EBF5
Actin
λ5
VpreB

Figure 3-3.

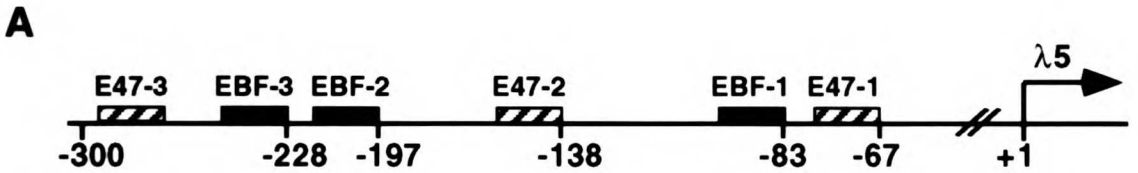
The λ5 enhancer/promoter region contains multiple EBF sites.

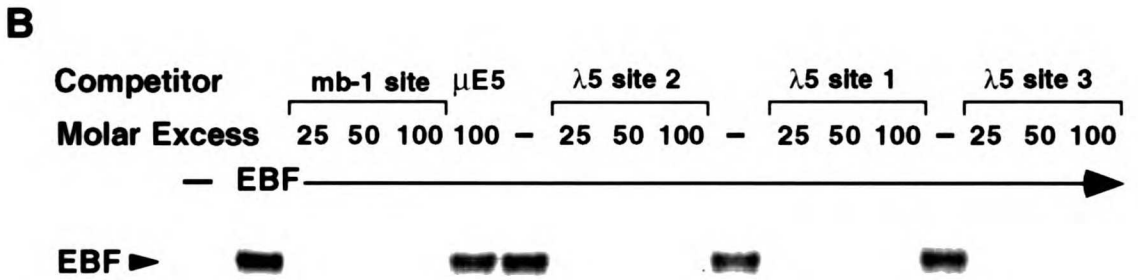
(A) Schematic diagrams of the λ5 enhancer/promoter region with the positions of the EBF binding sites (black boxes) and E-boxes (grey boxes) indicated. Numbers below the line refer to the nucleotide positions, assigning the transcription start site as +1 (Yang et al., 1995).

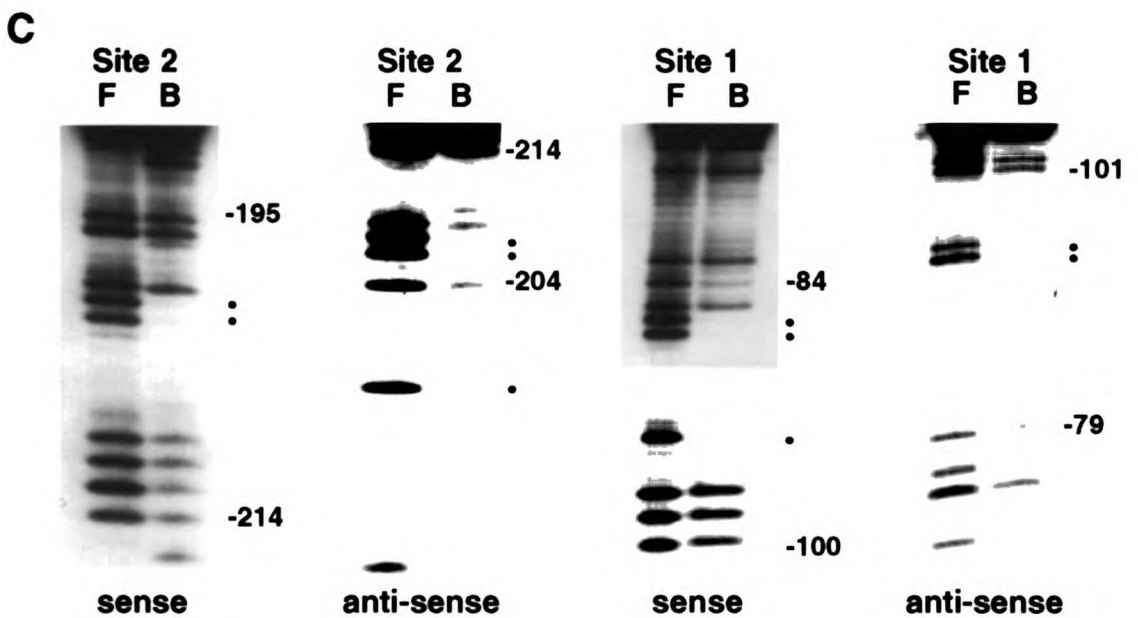
(B) Binding of EBF to the λ5 enhancer/promoter. Competition of the formation of the EBF:DNA complex by each of the three λ5 EBF binding sites in an electophoretic mobility shift assay. Lane 1 contains 0.5 μl unprogrammed rabbit reticulocyte lysate. All other lanes contain 0.5 μl EBF-programmed lysate incubated with a labeled mb-1 oligonucleotide containing an EBF binding site. 50, 100 or 200 fold molar excess of unlabeled duplex oligonucleotides was used for the competition of DNA binding by EBF as indicated. Unlabeled mb-1 and μE5 oligonucleotides were used for the competition as positive and negative controls, respectively.

(C) Methylation interference assay of EBF binding to sites 1 and 2 of the λ5 enhancer/promoter. Duplex oligonucleotides containing EBF-binding sites 1 or 2 were methylated at guanosines and incubated with recombinant in vitro-translated EBF. Bound [B] and free [F] DNA were separated in an electrophoretic mobility shift assay and analyzed on a sequencing gel. Methylated guanosines that interfere with EBF binding are indicated with a dot.

(D) Nucleotide sequences of the EBF binding sites in the λ5 enhancer/promoter.


A
E47-3
EBF-3
EBF-2
E47-2
EBF-1
E47-1
λ5
-300
-228
-197
-138
-83
-67
+1


B
Competitor
mb-1 site
μE5
λ5 site 2
λ5 site 1
λ5 site 3
Molar Excess
25 50 100 100 – 25 50 100 – 25 50 100 – 25 50 100
— EBF
EBF


C
Site 2
F B
-195
-214
sense
Site 2
F B
-214
-204
anti-sense
Site 1
F B
-84
-100
sense
Site 1
F B
-101
-79
anti-sense


D
EBF Site 3
AAGCCCTGGGGACC
TTC GGGACCCCTGG
-240
-228
EBF Site 2
AGGGGCCCTCAGGGACT
TCCCC GGGAGTCCCTGA
-213
-197
EBF Site 1
AGTCCTTTGGGAGA
TCAGGAAACCCTCT
-96
-83

Figure 3-4.

EBF binding sites are important for the activity of the λ5 enhancer/promoter in B cells.

(A) Electrophoretic mobility shift assay with nuclear extract from 230-238 pre-B cells. Five μg of nuclear extract were incubated with a labeled duplex oligonucleotide containing EBF site 1 of the λ5 promoter and the complexes were separated on a 6% polyacrylamide gel. The identity of the EBF:DNA complex was confirmed by reactivity with anti-EBF antiserum.

(B) Functional importance of the EBF binding sites in the λ5 enhancer/promoter region. The bar graph shows the results of transient transfections of 2 μg wild type or mutated □5 luciferase reporter genes into 230-238 pre-B cells. Luciferase activity was determined two days after transfection. Error bars indicate the maximal deviation from the mean value of four samples from two independent experiments. Deletions are indicated by a line and point mutations are represented by crosses.

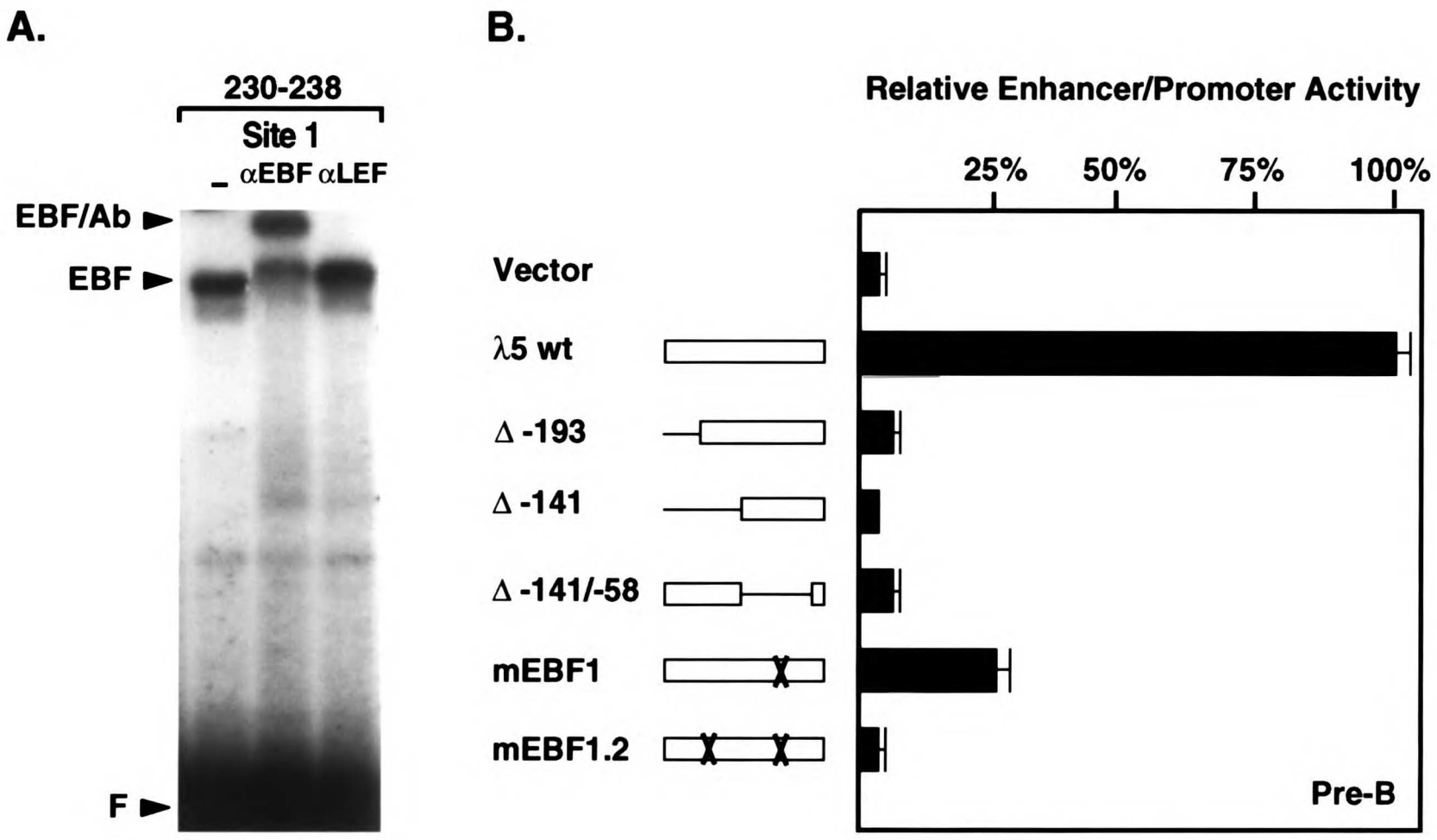

A.
230-238
Site 1
_ αEBF αLEF
EBF/Ab
EBF
F
B.
Relative Enhancer/Promoter Activity
25%
50%
75%
100%
Vector
λ5 wt
Δ -193
Δ -141
Δ -141/-58
mEBF1
mEBF1.2
Pre-B

Figure 3-5.

Covalent linkage of two E47 molecules results in an E47 forced dimer (E47FD) polypeptide that binds DNA efficiently.

(A) Schematic diagram of the fusion of two E47 molecules through a flexible linker. The basic helix-loop-helix (bHLH) DNA-binding domain is indicated.

(B) SDS-PAGE analysis of ^{35}S labeled *in vitro*-translated E47 and E47 forced dimer. The E47 forced dimer migrates with an apparent molecular mass of 120 kD.

(C) DNA binding by the E47 forced dimer. Electrophoretic mobility shift assay with unlabeled E47 and E47FD, which were translated in parallel to the reactions used for the SDS-PAGE analysis. The polypeptides were incubated with a labeled μE5 oligonucleotide in the absence or presence of anti-E47 or anti-T7 antibody.

(D) The E47 forced dimer binds the *λ5* enhancer/promoter region. Binding of E47FD to a labeled μE5 oligonucleotide in an electrophoretic mobility shift assay is competed by addition of excess of unlabeled *λ5* DNA fragment (nucleotides -299 to +131). The region of the gel containing the E47:DNA complex is shown.

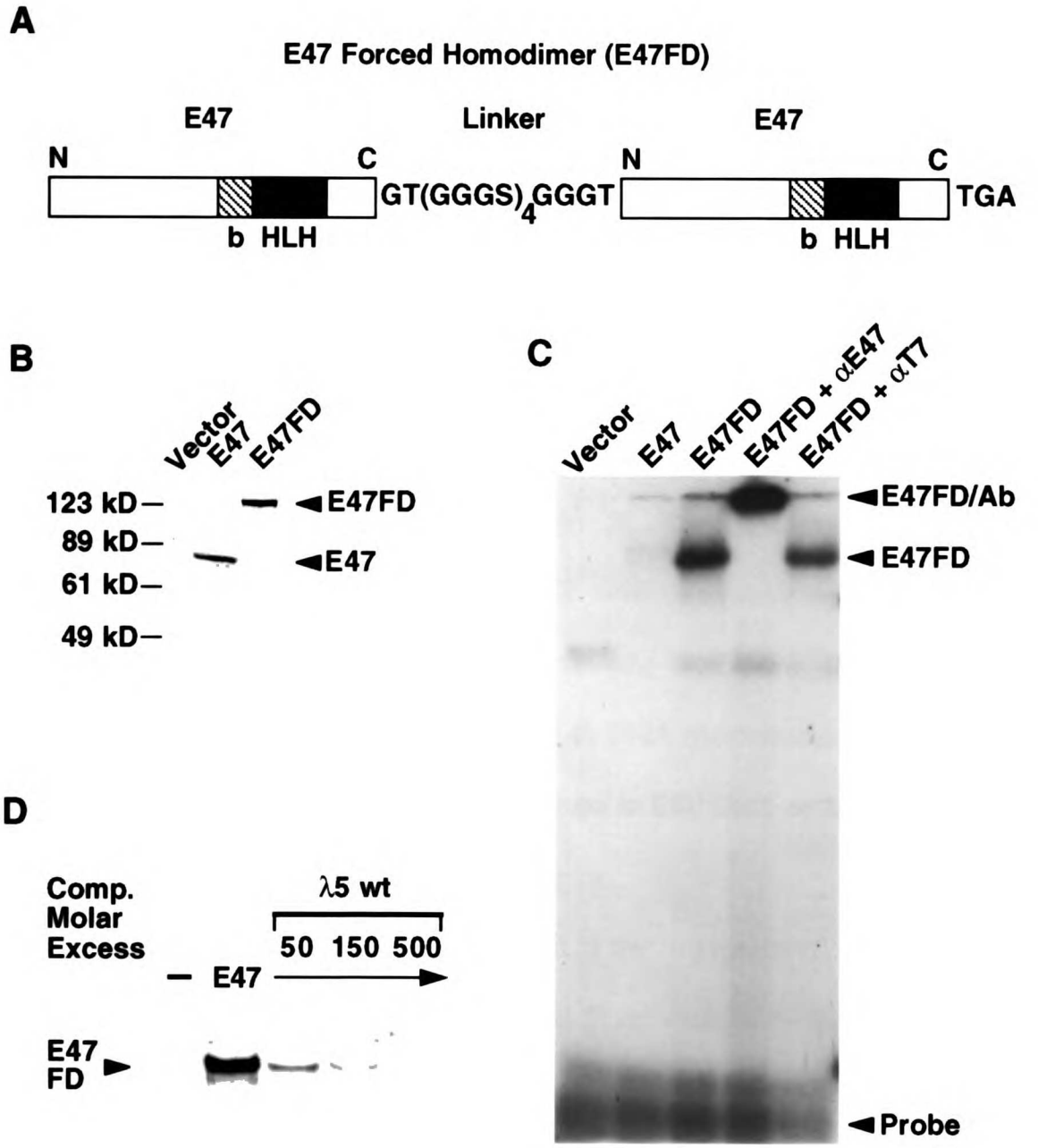

A
E47 Forced Homodimer (E47FD)
E47
Linker
E47
N
C
N
C
GT(GGGS)4GGGT
TGA
b HLH
b HLH
B
Vector
E47
E47FD
123 kD
89 kD
61 kD
49 kD
E47FD
E47
C
Vector
E47
E47FD
E47FD + αE47
E47FD + αT7
E47FD/Ab
E47FD
Probe
D
Comp.
Molar
Excess
λ5 wt
50 150 500
E47
E47
FD

Figure 3-6.

Synergistic activation of the λ5 promoter in Ba/F3 and Hela cells by EBF and E47.

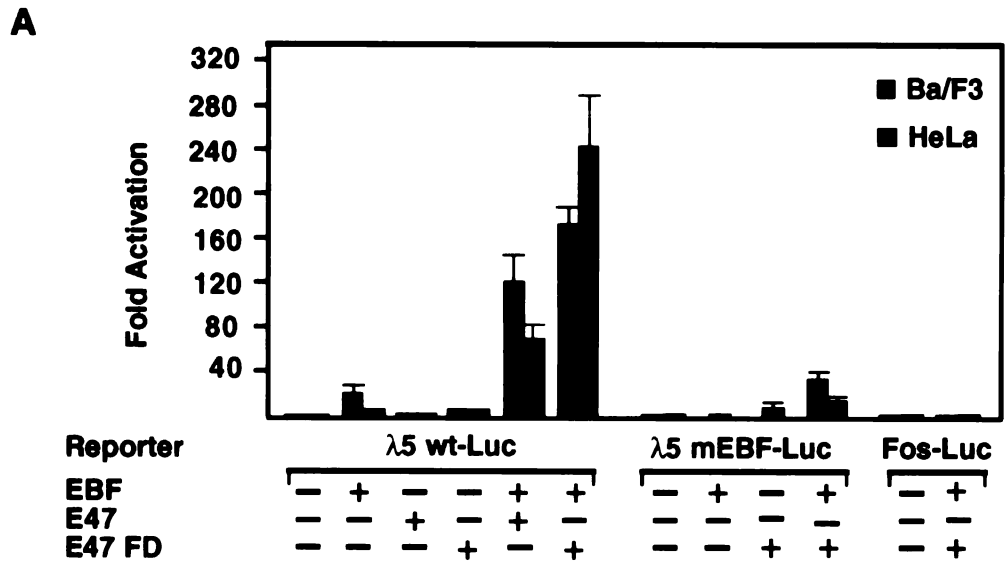
(A) Ba/F3 and Hela cells were transiently transfected with 0.5 μg of the λ5 luciferase reporter gene construct, containing nucleotides -299 to +131 of the λ5 gene, together with 250 ng EBF, 250 ng E47 or 250 ng E47FD cDNA expression plasmids alone or in pairwise combination as indicated. Fold induction was quantitated relative to the level of luciferase activity obtained with an expression vector lacking a cDNA insert. The λ5 mEBF luciferase reporter gene construct contains point mutations in all three EBF binding sites.

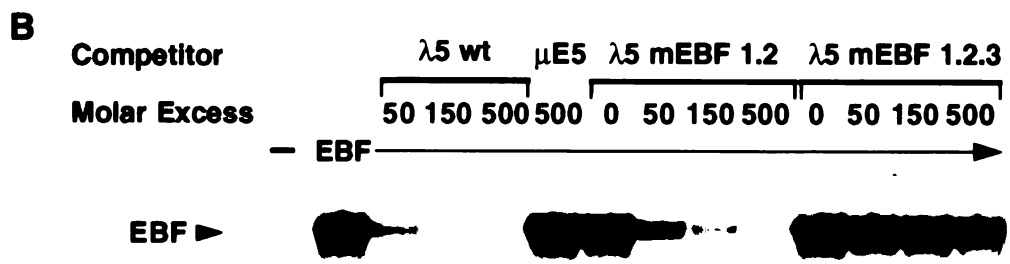
(B) Electrophoretic mobility shift assay of EBF binding to the λ5 enhancer/promoter. *In vitro*-translated EBF was incubated, with or without competitor DNA, with a labeled oligonucleotide containing the mb-1 EBF-binding site. For the competition, wildtype λ5 DNA (nucleotides -299 to +131) and mutant λ5 DNA containing point mutations in EBF site 1 or 2 or in all three EBF sites was used.

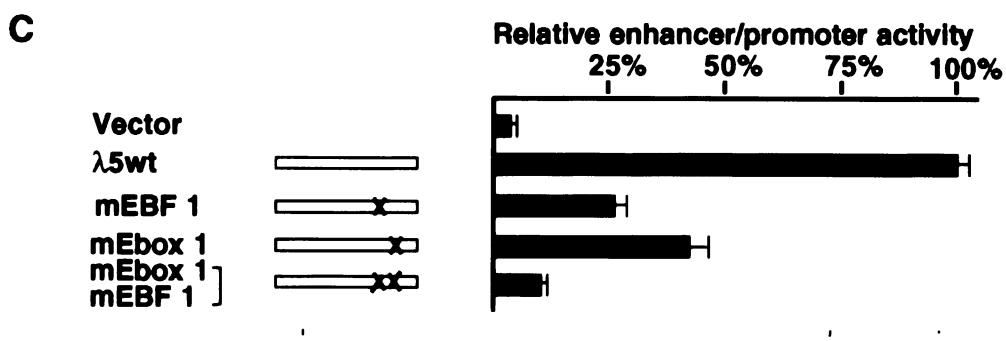
(C) Contribution of an E-box to the regulation of the λ5 promoter. Wild-type and mutated λ5 luciferase reporter gene constructs (2 μg) were transiently transfected into 230-238 pre-B cells. The mutations in the EBF site 1 and E-box 1 of λ5 enhancer/promoter (-299 to +131) are represented by crossed bars. The luciferase activity represents the average values obtained from four samples and two independent experiments.

Figure 3-6. (continued)

(D) Induction of endogenous *λ5* gene expression by transient ectopic expression of EBF and E47. Ba/F3 cells or NIH 3T3 cells were transiently transfected with 15 μg of the indicated expression plasmids. RNA was isolated after 48 hrs and analysed by RT-PCR analysis for expression of *actin* (20 cycles) and *λ5* (30 cycles for Ba/F3 cells and 35 cycles for NIH 3T3 cells).


A
320
280
240
200
160
120
80
40
Fold Activation
Ba/F3
HeLa
Reporter
λ5 wt-Luc
λ5 mEBF-Luc
Fos-Luc
EBF
E47
E47 FD


B
Competitor
λ5 wt
μE5
λ5 mEBF 1.2
λ5 mEBF 1.2.3
Molar Excess
50 150 500 500 0 50 150 500 0 50 150 500
EBF
EBF


C
Relative enhancer/promoter activity
25%
50%
75%
100%
Vector
λ5wt
mEBF 1
mEbox 1
mEbox 1
mEBF 1

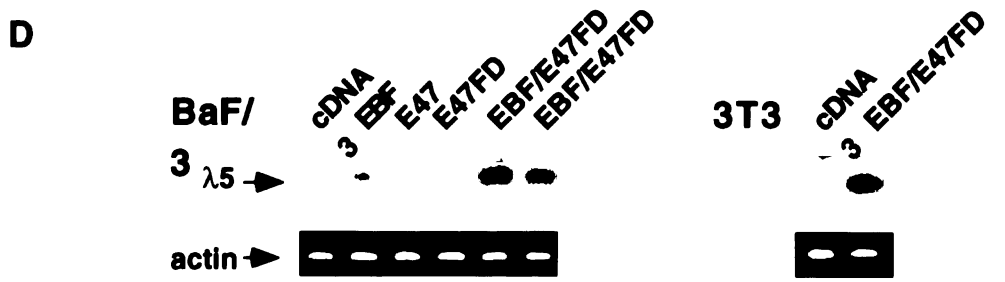

D
BaF/3
λ5
actin
cDNA
EBF
E47
E47FD
EBF/E47FD
EBF/E47FD
3T3
cDNA
EBF/E47FD

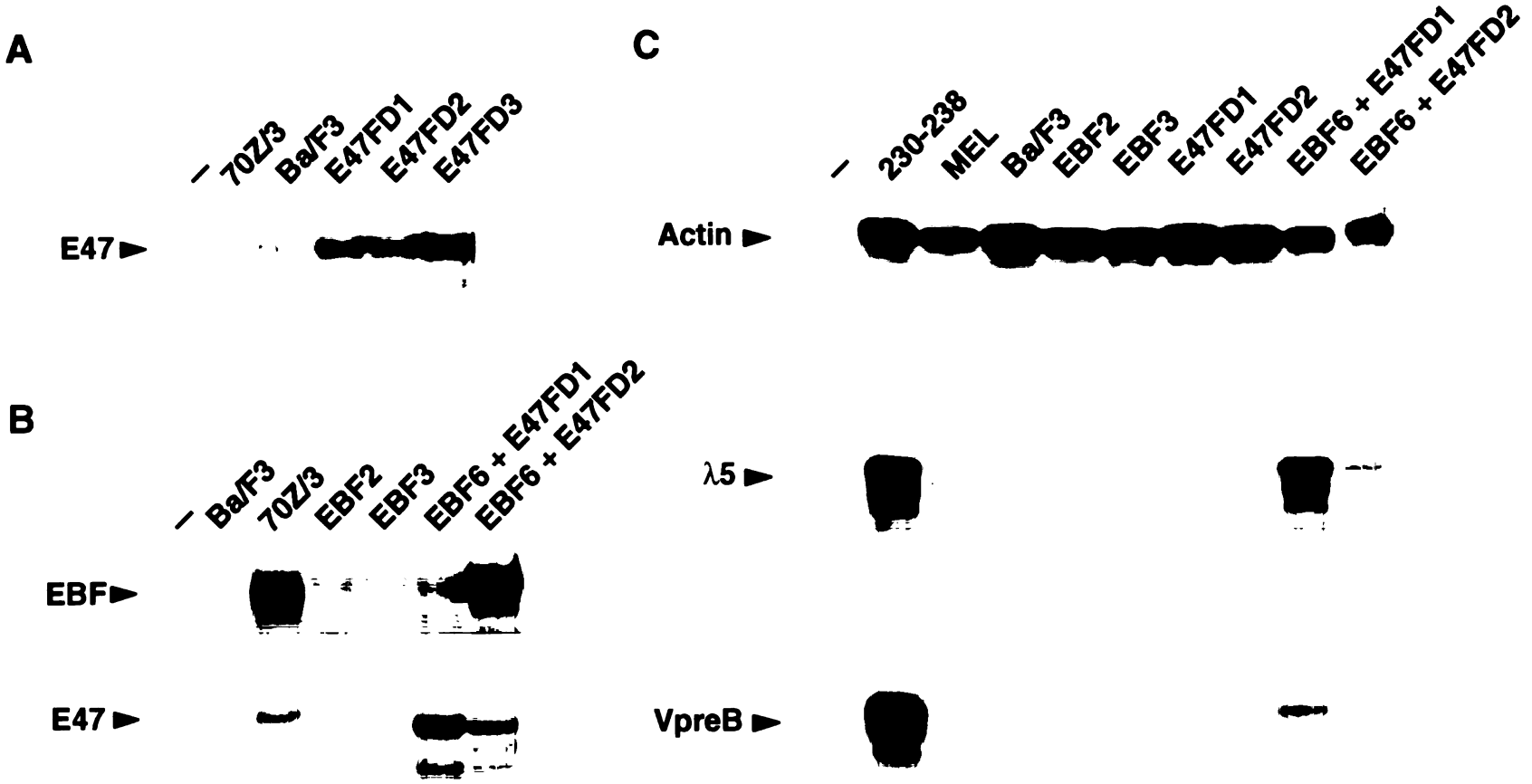
Figure 3-7.

Stable co-expression of E47FD and EBF induces high levels of $\lambda 5$ and V_{preB}.

(A) Electrophoretic mobility shift assay to detect E47 DNA binding activity in Ba/F3 cells transfected with E47FD cDNA. Five μg of nuclear extract was incubated with a labeled μE5 oligonucleotide probe.

(B) Electrophoretic mobility shift assay to detect E47 and EBF DNA-binding activity in Ba/F3 cells transfected with both EBF and E47FD cDNAs. Five μg of nuclear extracts from 70Z/3 pre B cells, untransfected and transfected BaF/3 cell clones were incubated with a labeled μE5 oligonucleotide (top panel) or a mb-1 oligonucleotide containing an EBF-binding site.

(C) EBF and E47 collaborate to promote high levels of transcription from the $\lambda 5$ and V_{preB} genes in stably transfected Ba/F3 cells. Autoradiograms show the results of S1 nuclease protection assays of total RNA from 230–238 pre-B cell, the parental Ba/F3 cells or Ba/F3 clones that have been stably transfected with EBF or E47FD alone or with both EBF and E47FD. Specifically protected fragments are detected in the 230–238 pre-B cells and in the Ba/F3 clones expressing both EBF and E47FD. The upper panel shows the results of the S1 nuclease protection analysis with an end-labelled β-actin probe and 5 μg total RNA. The lower panels show the S1 nuclease protection analysis with end-labelled $\lambda 5$ and V_{preB} probes and 25 μg and 10 μg total RNA, respectively.


A
– 70Z/3 Ba/F3 E47FD1 E47FD2 E47FD3
E47
B
– Ba/F3 70Z/3 EBF2 EBF3 EBF6 + E47FD1 EBF6 + E47FD2
EBF
E47
C
– 230-238 MEL Ba/F3 EBF2 EBF3 E47FD1 E47FD2 EBF6 + E47FD1 EBF6 + E47FD2
Actin
λ5
VpreB

CHAPTER 4

Coordinate Regulation of B Cell Differentiation by The Transcription Factors EBF and E2A

M.O'Riordan and R. Grosschedl. 1999. Coordinate Regulation of B Cell Differentiation by the Transcription Factors EBF and E2A. *Immunity* (in press).

Abstract

The transcription factors EBF and E2A are required at a similar step in early B cell differentiation. EBF and E2A synergistically upregulate transcription of endogenous B cell specific genes in a non-B cell line. Here we examine a genetic collaboration between these factors in regulating B lymphopoiesis. We find that *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice display a marked defect in pro-B cell differentiation at a stage later than observed in the single homozygous mutant mice. Pro-B cells from *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice show reduced expression of lymphoid specific transcripts, including *Pax5*, *Rag1*, *Rag2* and *mb1*. We also show that EBF directly binds and activates the *Pax5* promoter. Together, these data show collaboration between EBF and E2A and provide insight into the hierarchy of transcription factors that regulate B lymphocyte differentiation.

Introduction

The differentiation of B lymphocytes from hematopoietic progenitors is a complex and strictly regulated process. Stages of early B cell differentiation have been characterized by sequential rearrangement of the immunoglobulin (Ig) genes, and by the ordered appearance or disappearance of proteins at the cell surface (Rolink and Melchers, 1993). This program of differentiation involves the selective expression of genes that are characteristic of the B cell lineage. The correct temporal expression of these cell type-specific genes is determined by the action of lineage-restricted transcriptional regulators. Experiments using targeted gene disruptions have shown that the loss of some transcription factors, such as PU.1, affects multiple hematopoietic lineages, whereas the loss of other transcriptional regulators specifically affects early B cell development (Scott et al., 1994). These transcription factors include EBF (early B cell factor), E2A, and BSAP (*Pax5*) (Bain et al., 1994; Lin and Grosschedl, 1995; Reya and Grosschedl, 1998; Singh, 1996; Urbanek et al., 1994; Zhuang et al., 1994).

Ebf encodes a B cell specific transcription factor that binds DNA as a homodimer using a novel DNA binding domain and a dimerization domain related to the helix-loop-helix (HLH) motif of the basic-HLH family of transcription factors (Hagman et al., 1993; Hagman et al., 1995). Although the gene was named Early B cell Factor based on its cell type distribution, *Ebf* is also

expressed in brain, adipose tissue, and olfactory epithelium, where it was independently identified (*Olf-1*) (Hagman et al., 1993; Hagman et al., 1991; Wang and Reed, 1993). Targeted disruption of *Ebf* in mice results in animals with a severe defect in early B cell development, before any significant recombination of D_H-J_H segments has occurred in the immunoglobulin heavy chain locus (Lin and Grosschedl, 1995). Interestingly, *Ebf* heterozygous mice exhibit an approximately two-fold decrease in the number of cells in the pro-B lymphocyte compartment, indicating that normal B cell development depends on the presence of two wildtype *Ebf* alleles.

E2A, the founding member of the basic helix-loop-helix (bHLH) family of transcription factors, is expressed ubiquitously in two splice forms, E12 and E47, which differ in their bHLH domains (Murre et al., 1989). E2A binds a DNA sequence termed E-box (5' CANNTG), which is found in the promoters of many different cell type specific genes (Murre et al., 1994). Tissue specificity is, in part, determined by E2A dimerization partners, as described for the E47-MyoD interaction in myogenic differentiation (Lassar et al., 1991; Murre et al., 1989). A unique form of E2A exists specifically in B cells consisting of an E47 homodimer, termed BCF-1, or B Cell Factor-1 (Murre et al., 1991; Shen and Kadesch, 1995). Although E2A is widely expressed, targeted disruption of *E2a* produces viable animals that have a severe and specific defect in early B cell development, prior

to the onset of Ig gene rearrangement (Bain et al., 1994; Zhuang et al., 1994). *E2a* heterozygous mutant mice also exhibit an approximate two-fold decrease in the pro-B cell compartment.

The pronounced similarity of the B cell differentiation phenotype in *Ebf*$^{-/-}$ and *E2a*$^{-/-}$ mice led us to explore the possibility of cooperation between EBF and E2A in regulating a B-cell specific differentiation program. Ectopic expression of EBF and E2A in an immature hematopoietic cell line, Ba/F3, resulted in the transcriptional activation of the endogenous $\lambda 5$ and V_{preB} genes (Sigvardsson et al., 1997). The promoters of both the $\lambda 5$ and V_{preB} genes contain functional binding sites for EBF and E2A (Kudo et al., 1987; Sigvardsson et al., 1997). Strong transcriptional synergy was also observed in transient transfection assays, suggesting that EBF and E2A cooperate to directly activate at least two B cell specific targets. Although these results suggested a collaborative role for these transcription factors in the regulation of B cell specific genes in a tissue culture model, the experimental approach did not define a role for collaboration between EBF and E2A in normal B cell differentiation. Furthermore, we were unable to identify target genes for which EBF and E2A are necessary, but not sufficient for activation. The early arrest of B lymphocyte differentiation in the *Ebf* or *E2a* homozygous mutants also obscured the identification of additional target genes *in vivo*.

To examine the collaboration of EBF and E2A *in vivo,* we adopted a genetic approach analogous to synthetic lethality experiments in yeast. In this approach, hypomorphic alleles of two distinct genes are combined and the offspring analyzed for a compound mutant phenotype. A synthetic effect of the two mutations would suggest that the gene products act in the same genetic pathway. Here we show that *Ebf* and *E2a* act in the same genetic pathway by creating mice deficient in one allele of each gene. *Ebf/E2a* double heterozygous mice exhibit a marked defect in B cell differentiation. The analysis of these mice identifies new genetic targets of EBF and E2A, and reveals a collaboration between these factors in normal B lymphopoiesis.

Results

Analysis of Ebf/E2a double heterozygous mice reveals a defect in early B cell development

Based on previous observations that pro-B cell differentiation is dependent on the gene dosage of *Ebf* or *E2a,* we predicted that we would be able to detect a synthetic effect in mice heterozygous for mutations in both *Ebf* and *E2a* genes (Lin and Grosschedl, 1995; Zhuang et al., 1994). $E2a^{+/-}$ and $Ebf^{+/-}$ mice were crossed to generate litters that contained wildtype, *Ebf* and *E2a* single or double heterozygous pups. $Ebf^{+/-}E2a^{+/-}$ pups exhibited reduced postnatal

viability. Therefore, we examined B cell differentiation in fetal liver at E17.5 or E18.5, when double heterozygotes were represented in expected numbers.

Fetal liver cells were labeled with antibodies against the pan-B cell marker, B220, and CD43 (leukosialin), which is expressed on pro-B cells, and subjected to flow cytometry to determine the size of the pro-B cell compartment (Fig. 4-1A). This analysis revealed a marked defect in the numbers of cells in the pro-B compartment of the *Ebf/E2a* double heterozygous embryos compared to wildtype embryos. *Ebf* or *E2a* single heterozygous pups exhibited a two to three-fold decrease in pro-B cells relative to wildtype, consistent with previously published observations (Bain et al., 1994; Lin and Grosschedl, 1995; Zhuang et al., 1994). Analysis of multiple litters showed that *Ebf/E2a* double heterozygous fetal livers have, on average, a nine-fold reduction in the numbers of pro-B cells compared to wildtype littermates, and a four- to five-fold reduction relative to single heterozygous littermate controls (Fig. 4-1B). These data indicate that the transcription factors, EBF and E2A, cooperatively regulate early B cell development.

EBF/E2A double heterozygotes exhibit a defect in the transition of pro-B cells from Fraction B to Fraction C

The extensive molecular and phenotypic characterization of B cell differentiation allowed us to further define the defect of *Ebf/E2a* double heterozygous mice (Hardy and Hayakawa, 1995; Li et al., 1993). Fetal liver cells were stained with antibodies to B220, CD43, HSA (heat stable antigen) and a fourth marker, either BP-1 or CD19, and analyzed by flow cytometry (Fig. 4-2). The combination of these markers define pro-B cell populations as follows: the earliest population, fraction A, is $B220^+CD43^+HSA^-BP\text{-}1^-$; fraction B cells upregulate HSA to become $B220^+CD43^+HSA^+BP\text{-}1^-$; lastly, fraction C cells upregulate BP-1 to become positive for all four surface antigens.

As anticipated, *Ebf* and *E2a* single heterozygous mice displayed an approximate two-fold decrease specifically in the number of fraction B pro-B cells (Fig. 4-2A). This result is consistent with previous observations in homozygous single mutant mice that identified an absolute requirement for EBF or E2A in the transition from Fraction A to Fraction B (Bain et al., 1994; Lin and Grosschedl, 1995; Zhuang et al., 1994). The double heterozygous embryos showed a four-fold decrease in the numbers of fraction B cells, indicating an additive effect of the mutations in the *Ebf* and *E2a* genes. However, a significantly more pronounced decrease in the numbers of pro-B cells was observed in fraction C of the $Ebf^{+/-}E2a^{+/-}$ mice, in comparison to single heterozygotes (Fig. 4-2A). From these data, we conclude that EBF and E2A not

only regulate the transition from fraction A to B as previously shown, but act cooperatively to regulate further differentiation to fraction C. Thus, these experiments reveal two distinct requirements for EBF and E2A in pro-B cell differentiation.

To further characterize changes within fraction B that might indicate a problem in differentiation, we also analyzed the expression of CD19, considered a definitive marker of the B cell lineage (Rolink et al., 1996). This surface antigen is upregulated in fraction B pro-B cells and is a target of the transcription factor, BSAP (*Pax5*) (Kozmik et al., 1992; Nutt et al., 1998). Compared to littermate controls, *Ebf/E2a* double heterozygous mice have fewer $CD19^+$ cells, and a lower percentage of $CD19^+$ cells within Fraction B (Fig. 4-2B). This decrease in the number of CD19 expressing cells may be due to decreased expression of the *CD19* gene itself, or alternatively due to a defect in the differentiation of fraction B cells. These data corroborate our conclusion that EBF and E2A are coordinately required for normal differentiation of fraction B to fraction C.

Adult $Ebf^{+/-}E2a^{+/-}$ animals show a defect in bone marrow B cell development

Ebf/E2a double heterozygous mice that survived to adulthood were used to determine whether or not the fetal liver B cell defect was recapitulated in adult B lymphopoiesis. Analysis of bone marrow cells from four-month old mice with

antibodies against B220 and IgM revealed that the $B220^{lo}IgM^{-}$ population, which includes pro-B and pre-B cells, was significantly decreased in $Ebf^{+/-}E2a^{+/-}$ mice (Fig. 4-3A). The numbers of $B220^{hi}IgM^{+}$ recirculating B lymphocytes are also reduced, although analysis of splenocytes clearly shows the presence of B cells (Fig. 4-3A and B). In a separate experiment, analysis of the spleen revealed that $B220^{+}$ lymphocytes in $Ebf^{+/-}E2a^{+/-}$ mice express both surface IgM and IgD (data not shown), suggesting that a certain percentage of developing B cells can progress to maturity and exit into the periphery. These data indicate that bone marrow B cell development is also affected in *Ebf/E2a* double heterozygous mice.

Expression levels of EBF and E47, but not E12, transcripts are constant in pro-B cells

One possible explanation for the differential defect between the first (fraction A to B) and second (fraction B to C) pro-B cell transitions would be a requirement for higher levels of EBF and E2A in fraction B. To examine the expression levels of these genes during B cell differentiation, wildtype E17.5 fetal liver cells were labeled with antibodies against B220, CD43, HSA and BP-1, and sorted by flow cytometry into three different pro-B cell fractions, A, B and C. The cDNA was normalized relative to the levels of actin transcripts and amplified in a linear range as shown for *IL7R* (Fig. 4-4A). Levels of *Ebf* and *E47* transcripts remained constant in pro-B cell fractions A, B and C, while levels of

E12 transcripts increased from fraction A to B (Fig. 4-4B). If a requirement for higher levels of E12 caused the marked block in the transition of fraction B to C in the *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver, we might also expect to detect a similar block of differentiation in the *E2a*$^{+/-}$ fetal liver as well, which we did not observe (Fig. 4-2A). Therefore, the additional developmental defect in the fraction B to C transition in *Ebf*$^{+/-}$*E2a*$^{+/-}$ mice is unlikely to be due to differential expression levels of either EBF or E2A. This developmental defect could, however, reflect a pronounced synergy in the function of EBF and E2A at this stage of differentiation or, alternatively, a collaboration of both transcription factors with another transcription factor.

Expression of critical genes in Fraction B is decreased in Ebf/E2a double heterozygotes

To understand the molecular basis of the B cell deficiency in *Ebf/E2a* double heterozygous mice, we examined the expression of multiple genes known or presumed to regulate B cell development. Toward this end, we performed RT-PCR analysis on B220^{+} cell populations that were sorted from the fetal liver of E15.5 embryos using magnetic beads (Fig. 4-4D, Table 1). At this stage of mouse embryogenesis, very few pro-B cells have progressed to fraction C, as defined by expression of the surface markers, BP-1 and HSA (Fig. 4-4C). To control for a similar composition and number of sorted B cells, we normalized the cDNA

samples using RT-PCR to detect IL7 receptor (*IL7R*) transcript levels, which were similar in FACS-sorted fraction B cells of all genotypes (data not shown). Moreover, *IL7R* gene expression was previously shown to be unaffected in the *Ebf* homozygous mutant (Lin and Grosschedl, 1995). Aliquots of RT-PCR reactions were withdrawn at multiple cycles to ensure amplification in the linear range (data not shown).

Analysis of the previously identified targets for EBF and E2A, $\lambda 5$ and V_{preB}, revealed a significant reduction in $Ebf^{+/-}E2a^{+/-}$ fetal liver samples, but not in wildtype, *Ebf* or *E2a* single heterozygous controls (Fig. 4-4D). We also examined expression of the *Pax5* gene, which has been shown to control the transition from fraction B to C in adult bone marrow B cell development (Nutt et al., 1997; Urbanek et al., 1994). We found that in double heterozygous pro-B cells, *Pax5* expression was significantly decreased, suggesting that *Pax5* is genetically downstream of both *Ebf* and *E2a*. Other genes expressed at a reduced level in $Ebf^{+/-}E2a^{+/-}$ pro-B cells include the recombination activating genes, *Rag1* and *Rag2*. These data suggest that *Rag* gene expression, found in both B and T lymphocytes, is coordinately regulated by B cell specific transcription factors, raising the possibility that these genes are regulated distinctly in the lymphoid lineages. The introduction of a rearranged immunoglobulin transgene, encoding a HEL specific heavy and light chain, was not able to rescue the defect in $Ebf^{+/-}E2a^{+/-}$ pro-

B cell development (data not shown) as was observed for *Rag*$^{-/-}$ mice (Goodnow et al., 1988; Mason et al., 1992; Chen et al., 1994; Spanopoulou et al., 1994). *Mb1* and *B29*, the signaling components of the surface immunoglobulin complex were differentially affected by the loss of one *Ebf* and one *E2a* allele. *Mb1* was expressed at a reduced level in *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B cells, whereas *B29* transcription was only modestly affected. *B29* transcription has been observed in hematopoietic cell types other than B cells, whereas *mb1* expression is confined to the B-cell lineage (Wang et al., 1998).

We also examined the expression of other candidate target genes known to be expressed in fraction B cells that are involved in regulating gene expression, cell cycle progression, signaling and cell survival (Table 1). Among these, the transcription factor genes, *E12, Lef1*, and *Sox-4*, and the *CD19* gene were found to be modestly downregulated in *Ebf*$^{+/-}$*E2a*$^{+/-}$pro-B cells. *Lef1* and *CD19* are downstream targets for BSAP, and may only indirectly regulated by EBF and E2A (Nutt et al., 1998). Taken together, these data identify a number of new genetic targets for the coordinate function of EBF and E2A in the development of the B cell lineage (summarized in Table 1).

The observed reductions in the RT-PCR assay on bulk sorted cell populations could reflect fewer pro-B cells expressing the target transcripts, or a reduction in the level of target gene transcription in all of the *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B

cells. To distinguish between these two possibilities, we sorted several B220$^+$BP-1$^-$ cells from either wildtype or *Ebf*$^{+/-}$*E2a*$^{+/-}$ fetal liver into each well of a 96-well plate, and performed RT-PCR on these samples using primers for V_{preB} or *Rag-2* (Fig. 4-4E, Table 1). In *Ebf*$^{+/-}$*E2a*$^{+/-}$ samples, we observed a four- and seven-fold decrease respectively in the number of wells yielding a positive RT-PCR signal for V_{preB} or *Rag-2*, compared to wildtype. All wells shown were positive in an actin RT-PCR assay (data not shown). This analysis confirms the differences seen in the bulk RT-PCR assay, and further suggests that a lower dose of both transcription factors reduces the frequency of gene activation in a cell population, rather than lowering the level of gene activation in all cells.

EBF and E47 form a ternary complex on sequences from the λ5 enhancer/promoter

The observation that EBF and E2A act coordinately in B cell development led us to examine whether this functional collaboration *in vivo* can be accounted for by cooperative DNA binding of these transcription factors. We had previously identified multiple EBF- and E2A-binding sites in the *λ5* enhancer/promoter, that were important for transactivation by EBF and E47 (Sigvardsson et al., 1997). This finding suggested that cooperation between EBF and E2A could, at least in part, be mediated directly at the promoter. To look for the formation of a ternary complex, we performed an electrophoretic mobility

shift assay with sequences from the λ5 promoter with *in vitro* translated EBF and E47 proteins (Fig. 4-5). Both proteins were able to bind to the λ5 sequence individually. Addition of increasing amounts of EBF added to E47 resulted in the formation of a slower migrating complex. This complex could be competed away by the addition of excess unlabeled oligonucleotides containing either an EBF- or E2A- binding site, suggesting that both EBF and E2A proteins were present in the complex. We therefore conclude that EBF and E47 are able to form a co-complex using physiological binding sites, which may contribute to the cooperativity observed *in vivo*.

EBF binds and activates the B cell specific Pax5 promoter

Our observations of coordinate regulation of the λ5 gene encouraged us to study a potentially more relevant target of EBF and E2A, the *Pax5* gene. The expression of *Pax5*, as well as the disruption of B cell development in *Pax5*$^{-/-}$ mice, are consistent with the *Pax5* gene being regulated by EBF and/or E2A (Adams et al., 1992; Urbanek et al., 1994). Furthermore, expression of E12 in a macrophage-like cell line results in the expression of both *Ebf* and *Pax5* transcripts (Kee and Murre, 1998). However, this study did not determine whether the *Pax5* gene is a direct target of either EBF or E2A. We therefore cloned 1.8 kilobases of sequence upstream of the murine B cell-specific start site in exon 1A of the *Pax5* gene

(Busslinger et al., 1996). Analysis of the promoter identified multiple E-box sequences, but none tested were found to bind to either *in vitro* translated E12 or E47. A putative EBF site (*Pax5-EBF1*) was identified 1121 base pairs upstream of the transcription start site and was used as a probe in an electrophoretic mobility shift assay (Fig. 4-6A). *In vitro* translated EBF bound to the radioactively labeled *Pax5-EBF1* site, and was supershifted by anti-EBF antisera. The EBF:DNA complex could be competed away by specific but not non-specific unlabeled competitor oligonucleotides. These data suggest that EBF may regulate *Pax5 in vivo* by binding directly to the promoter.

In order to test the function of the promoter, the 1.8 kb *Pax5* genomic fragment was cloned into a luciferase reporter construct. NIH3T3 cells were transiently transfected with the reporter construct together with plasmids expressing EBF or E2A (Fig. 4-6B). EBF was able to transactivate the *Pax5* promoter up to eighteen-fold over the promoter alone. E2A did not transactivate the *Pax5* promoter in this assay, consistent with a lack of functional E2A binding sites in the 1.8 kb fragment. Furthermore, E2A and EBF together did not activate to a greater degree than EBF alone. A covalently linked homodimer of E47 acted indistinguishably from E47 in this assay (data not shown). The decrease in activation seen in EBF/E2A combinations is most likely due to a non-specific toxicity to the cells by the E2A proteins. All samples were normalized to the

expression of CMV-βgalactosidase, however, we could not completely titrate out the observed non-specific toxicity of the E2A proteins. Although we were unable to show direct regulation of the *Pax5* promoter by E2A, additional *cis*-regulatory elements may be required to allow the normal function of this promoter in B cells.

Discussion

Our experiments show that the loss of one allele each of *Ebf* and *E2a* markedly affects the differentiation of pro-B cells, compared to single heterozygous mice. Notably, the defect in double heterozygous mice occurs later in differentiation than that observed in single homozygous mutant mice. Moreover, transcription of multiple lymphocyte specific genes is significantly affected in $Ebf^{+/-}E2a^{+/-}$ mice. We show that EBF and E2A can form a ternary complex at a target promoter, suggesting a possible contribution to functional cooperativity at the level of DNA binding. Finally, we show that EBF can directly bind and regulate the *Pax5* promoter. Together, these data suggest that coordinate regulation by distinct transcription factors is important in establishing temporal patterns of gene activation during B cell differentiation.

Cell type specific gene expression can be achieved, in principle, by transcriptional regulators that are dedicated to a given cell type, or by the

overlapping expression of transcription factors found in multiple cell types. Combinatorial control of differentiation has been well established for myogenesis in which two muscle specific transcription factors, MEF2 and the bHLH protein, MyoD, cooperate to regulate specific gene expression (Molkentin et al., 1995). However, in B cell differentiation, no such collaboration between distinct transcription factors has been described to account for cell type specific expression. The E2A gene products, E12 and E47, are expressed ubiquitously, although the homodimeric form of E47, termed BCF-1, is found only in B cells (Murre et al., 1991; Shen and Kadesch, 1995). Recent data suggest that formation of the E47 homodimers is regulated by the formation of intermolecular di-sulfide bonds and by protein dephosphorylation specifically in B cells (Benezra, 1994; Sloan et al., 1996). Conversely, the expression pattern of EBF is restricted to B cells and several other non-lymphoid cell types (Garel et al., 1997; Hagman et al., 1993; Wang and Reed, 1993). Ectopic expression of E47 alone in non-B cell lines, does not result in the activation of B cell specific genes, although E47 has been shown to weakly induce the expression of lymphoid genes such as *Rag-1, Rag-2* and *TdT* (Choi et al., 1996; Schlissel et al., 1991). Likewise, ectopic expression of EBF in non-B cells only weakly activates B cell specific genes (Sigvardsson et al., 1997). In combination, however, EBF and E47 synergize to activate high levels of expression of two pro-B cell specific genes, $\lambda 5$ and V_{preB} in non-B cell lines. As

would be predicted, the $\lambda 5$ and V_{preB} genes were expressed in $Ebf^{+/-}E2a^{+/-}$mice at very low levels, showing the synergy between EBF and E2A in an *in vivo* model.

Transcriptional synergy may be achieved by multiple mechanisms. First, DNA binding proteins may bind cooperatively at a target promoter. We have been able to observe a co-complex on $\lambda 5$ enhancer/promoter sequences, containing both EBF and E47. However, we have been unable to show any direct physical interaction between EBF and E2A, in co-immunoprecipitation experiments using either *in vitro* translated proteins, or B cell extracts (data not shown). It is possible that stable association of the two proteins may require an intermediary protein, as found in the association of the transcriptional co-activator, ALY with LEF-1 and AML-1, which regulate the TCRα enhancer (Bruhn et al., 1997).

Second, transcription factors can exhibit a functional synergy without physical interaction with each other, possibly by contacting components of the general transcription or chromatin remodeling machinery (Hernandez-Munain and Krangel, 1995). Consistent with this view, previous experiments using a cell line, stably transfected with EBF or E47, showed that EBF alone, but not E47, could activate the endogenous $\lambda 5$ locus to a low level, although E47 has strong activating potential in transient transfection assays (Sigvardsson et al., 1997). In combination, both factors induced expression of the endogenous $\lambda 5$ locus to

levels approaching those found in a pro-B cell line. These data suggest that the observed functional synergy may in part be due to the ability of EBF to bind its target in nuclear chromatin, perhaps making the locus available to the activating function of E2A. Finally, the *λ5* promoter sequence contains multiple EBF binding sites and E-boxes that may be important for the synergistic function of these transcription factors. In the physiological process of B cell differentiation, functional cooperativity of EBF and E2A in activating target genes may be a combination of any of these mechanisms.

Finally, a possible mechanism for synergy might depend on the amount, or dose, of activators present. It had been previously observed that mice heterozygous for *Ebf* or *E2a* exhibit an approximately two-fold decrease in the number of cells in the pro-B cell compartment, and the B cells were, therefore, sensitive to the gene dosage of *Ebf* or *E2a* (Lin and Grosschedl, 1995; Zhuang et al., 1994). Several examples of dose dependence have been described in mice in which a null mutation in one allele results in a mutant phenotype that is intermediate between the wildtype and homozygous mutant phenotypes, such as mice deficient in the gene encoding the transcriptional co-activator, p300 (Yao et al., 1998). At a molecular level, several mechanisms could account for the dose dependence of a gene encoding a transcription factor. The multiplicity of transcription factor binding sites in promoters, such as the *λ5* promoter, might

impose dose dependence if occupancy of all binding sites is required for a transcriptional response. Similarly, the heterozygosity of a gene encoding a rate-limiting factor in the assembly of a multi-protein complex would decrease formation of the functional complex (Henikoff, 1996). The appearance of a dose dependent phenotype in mutant mice could also be the result of monoallelic expression of a critical transcription factor. Random inactivation of a critical gene in heterozygous mutant mice would confer a null phenotype on fifty percent of the cells. Recent data show that the *Pax5* gene is expressed in a monoallelic manner (Nutt et al., 1999). This is not the case for the *E2a* locus, since mature B cells from mice containing one *E2a*βgalactosidase knock-in allele express both the wildtype E2A protein and the E2Aβgal fusion protein in the same cell (Zhuang et al., 1994). However, the possibility of monoallelic expression remains for the *Ebf* locus.

The specific stage at which pro-B cells from $Ebf^{+/-}E2a^{+/-}$ animals are deficient is consistent with a critical defect in transcription of either the *mb-1* or the *Pax5* genes, both of which affect the transition from fraction B to fraction C (Torres et al., 1996; Urbanek et al., 1994). This defect is earlier than the B cell developmental arrest observed in $Rag1^{-/-}$ and $Rag2^{-/-}$ mice (Mombaerts et al., 1992; Shinkai et al., 1992). Although *Rag1* and *Rag2* are also expressed in T lymphocytes, our observation that expression of the Rag genes is reduced in

Ebf/E2a double heterozygous mice strongly suggests these genes are distinctly regulated in B and T cells by lineage specific factors, such as EBF or BCF-1. BSAP, a B lineage restricted transcription factor encoded by *Pax5,* is known to affect the expression of genes such as *CD19, mb1, Lef1,* and *N-myc,* and binding sites for BSAP have been identified in the promoters of many other B cell-specific genes, including $\lambda 5$ and V_{preB} (Kozmik et al., 1992; Nutt et al., 1998; Tian et al., 1997). The requirement for BSAP in B cell differentiation in fetal liver is earlier than in adult bone marrow, since no $B220^+$ cells are observed in the fetal liver of *Pax5* null mice (Nutt et al., 1998; Nutt et al., 1997). However, recent data suggest that the activity of BSAP is concentration dependent, indicating that some functions of BSAP may be more susceptible to changes in BSAP levels than others (Wallin et al., 1998). In the functionally hypomorphic *Ebf/E2a* double heterozygotes, enough BSAP may be expressed to allow commitment to the B cell lineage but an insufficient amount to regulate the transition from fraction B to C. Furthermore, both EBF and BSAP are regulators of the *mb-1* gene (Fitzsimmons et al., 1996; Hagman et al., 1991). Low levels of functional Igα, encoded by *mb1,* may be insufficient to transduce important developmental signals. It has been reported that BSAP also regulates B cell proliferation (Wakatsuki et al., 1994). Likewise, EBF orthologs may be involved in cell cycle control, since the expression of *String,* a *cdc25*-like cell cycle regulator, in mitotic

domain 2 coincides with the expression of *collier*, the Drosophila ortholog of EBF (Crozatier et al., 1996). However, we were unable to observe any change in the expression of the *CDC25* gene family members expressed in B lymphocytes (data not shown). Given the striking conservation between *collier* and *Ebf*, we would predict that some target genes would be conserved. It is intriguing to speculate that one conserved function of *Ebf* family members may be to regulate specific *Pax* genes. Our observations support a direct role for EBF in the activation of *Pax5*. Although we have not found any evidence for regulation of the *Pax5* promoter fragment by E2A, it is likely that another regulatory element, such as an enhancer, is also required for the proper expression of the *Pax5* gene that requires the activity of E2A and/or EBF. It has been reported that twelve kilobases of 5′ flanking sequence can direct high level B cell expression, although no specific regulatory regions have yet been characterized (Busslinger and Nutt, 1998). The finding that *Pax5* expression is coordinately regulated by EBF and E2A, as shown by our genetic experiments, suggests a hierarchy among transcription factors that are known to be involved in many different development processes (Mansouri et al., 1996; Murre et al., 1994; Wehr and Gruss, 1996).

The process of B cell differentiation appears to require EBF and E2A at different stages of development. The transcriptional synergy of EBF and E2A *in*

vivo may be important in establishing a specific temporal expression pattern within the B cell lineage. Temporal regulation of the *Pax5* gene by EBF and E2A may be of particular importance as recent studies have implicated *Pax5* in commitment to the B cell lineage (M. Busslinger, personal communication). Both EBF and E2A are expressed at the earliest stages of B cell differentiation and regulate target genes at that stage. One of these target proteins may then facilitate the functional collaboration between EBF and E2A, which in concert would regulate target genes required at a later stage of differentiation. In particular, for the regulation of the transition of fraction B to C of pro-B cell development, the synergistic action of EBF and E2A may also involve collaboration with *Pax5*. Such a mechanism that is dependent on the collaboration of multiple factors would increase the accuracy of temporal regulation of gene expression. Our *in vivo* cooperativity studies have not only identified some of the downstream genes for which both EBF and E2A are necessary, but have also defined a novel physiological requirement for these two transcription factors in B cell development. Further studies will be required to pinpoint additional targets of EBF and E2A that promote progression through the early stages of B lymphocyte differentiation.

Materials & Methods

Mice

E2aβgal$^{+/-}$ mice on a 129/Sv background were bred to *Ebf*$^{+/-}$ mice on a C57BL/6J background to generate litters for RNA and FACS analysis. Animals used for most experiments were bred in a non-barrier facility. Mice were genotyped by PCR using the following conditions: *E2a* PCR 94° C for 30 sec, 60° C for 30 sec, 72° C for 1 min for 30 cycles, *Ebf* PCR 94° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec for 30 cycles. The following primers were used for the genotyping PCR reactions: *E2a* PCR (1) 5′ CCGAGCTCCTTAAAGGCCTCA, (2) 5′ TTGTGGACATTTTCTAGGCAG, (3) 5′ GTTGTGCCCAGTCATAGCCG (Y. Zhuang); *Ebf* PCR (1) 5′ GCCAACAGCGAAAAGACC (2) 5′ GGAGCCTCACCATTGCTGTAGAG, (3) 5′ ATGGCGATGCCTGCTTGCCGAATA. Ig heavy + light chain (MD4) transgenic mice and primers were provided by J. Cyster. PCR primers for MD4 genotyping are as follows: IgH_{F1} 5′ GCGACTCCATCACCAGCGAT, IgH_{F2} 5′ CTGGAGCCCTAGCCAAGGAT, IgH_{R1} 5′ ACCACAGACCAGCAGGCAGA.

Flow cytometry

Fetal liver or bone marrow cells were collected in cold FACS buffer (1XPBS + 2% fetal calf serum) and homogenized with a syringe. Cells were layered over Lympholyte M (Cedarlane) and centrifuged to remove erythrocytes and cellular

debris. Cells remaining at the interface were collected and washed with FACS buffer. The cells were then incubated with primary antibodies for 15 min. on ice. For four-color analyses, cells were washed after primary staining, and then incubated with streptavidin-Red613 (Gibco BRL) for 15 min. on ice. The cells were washed one final time before resuspension in preparation for flow cytometric analysis. All analysis was done using Cellquest software (Becton-Dickinson). All of the antibodies used in flow cytometry experiments were obtained from PharMingen.

RT-PCR

Fetal liver cells were either magnetically sorted with beads coated with anti-mouse B220 antibody (Dynal) or FACS sorted (FACStar; Becton Dickinson). Each sample shown represents one fetal liver; no samples were pooled. For the 2-cell RT-PCR experiment, cells were sorted into 96-well plates (MJ Research) containing RT lysis buffer as previously described (Klug et al., 1998). RNA for bulk RT-PCR was purified with Trizol (Gibco BRL). The samples were treated with RQ1 DNase (Promega) before inactivation by phenol/chloroform extraction and ethanol precipitation. The fetal liver bulk RNA samples were then reverse transcribed with MMLV-RT (Gibco BRL). The cDNA reactions were brought up to a final volume of 100 μl and 5 μl used in an RT-PCR reaction. All bulk RT-PCR

reactions were standardized by levels of β-actin or by levels of IL7 receptor. Standard RT-PCR conditions were as follows: 94° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec, using 0.1 μl of Taq polymerase (Boehringer Mannheim) per reaction for the number of cycles indicated in the figure legend. Aliquots were withdrawn at 27, 30 and 33 cycles to determine the minimum amplification necessary for detection. Primers used for bulk RT-PCR reactions have been described previously except for the following which were designed using Geneworks software (Lin and Grosschedl, 1995; Wasserman et al., 1995): Cdc25A (Genbank accession # U27323) (1) 5′ CAGAATCGACCGAATCAGG, (2) 5′ TGTCCAGAGGCTTACCATGC; Cdc25L (L16296) (1) 5′ CCACTCTGTGACAT GAACGC, (2) 5′ CAGAGGAACAACAGGCTTCC; Cdc25B (D16237) (1) 5′ CAAGAATGCTGTGAACCTGC, (2) 5′ TGGTTCATGGGTCGGTAGTC; Tcf4 (IMAGE clones 444295/764951) (1) 5′ CCAAGCAGGAAGCCTCCAGAGC, (2) 5′ GAGGGACCATATGGGGAGGGAA; Sox4 (X70298) (1) 5′ GGAGGCCCGGG ATGCTCGCCCGATG, (2) 5′ GTCCGGGAATTCGAAGTGGGAGCCT; Lef1 (X58636) (1) 5′ CTCCTGTAGCTTCTCTCTCTTCC, (2) 5′ CAACACGAAC AGAGAAAGGAGCA; $BclX_L$ (X83574) (1) 5′ TGCGTGGAAAGCGTAGA CAAGG, (2) 5′ AGTGGATGGTCAGTGTCTGGTCAC; Ikaros VI (S74708) (1) 5′ ACGAATGCTTGATGCCTCG, (2) 5′ TTGTGAGGCTTACCAACGG; CD19 (M62542) (1) 5′ AAGGAAGCGAATGACTGACC, (2) 5′ TCTGAGCTCCAGTATC

CTGG. Nested RT-PCR primers were used, except for actin, for the 2-cell PCR reactions as follows: V_{preB} outer primers (X05556) (1) 5′ TGGCCTATCTCACAGGTTGTG, (2) 5′ ACGGCACAGTAATACACAGCC, V_{preB} inner primer (used with primer 1) (3) 5′ GAAGTATCTCAGCAGGAACCTGG; Rag-2 outer primers (M64796) (1) 5′ CACATCCACAAGCAGGAAGTACAC, (2) 5′ TCCCTCGACTATACACCACGTCAA, Rag-2 inner primers (3) 5′ CCTGCAGATGGTAACAGTGG, (4) 5′ TCTTGCAA CGACAGACATG; Actin (X03765) (1) 5′ GACGACATGGAGAAGATCTGG, (2) 5′ TGTGGTGGTGAAGCTGTAGC. V_{preB} primer 2, Rag-2 primer 2 and Actin primer 2 were included in the RT lysis buffer as RT primers. Three μl of the 20 μl RT reaction was used in the first round of PCR with the outer primer set (primers 1 and 2) for 30 cycles of 94° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec, using 0.1 μl of Taq polymerase per reaction. One μl of the first round reaction was used in the second round of PCR with the inner primer (V_{preB} primers 1 and 3; Rag-2 primers 3 and 4) set for 30 cycles, using the same conditions as the first. One round of 40 cycles using the actin PCR primers was performed using the conditions described above, as a sorting control. All PCR reactions were run on 2% agarose gels with ethidium bromide, and visualized by UV transillumination. Images were captured with a gel documentation system (Alpha Innotech).

Electrophoretic mobility shift assays

EBF and E47 cDNAs were transcribed and translated *in vitro* using the TNT Reticulocyte Lysate coupled transcription/translation system (Promega). The proteins were added together with unprogrammed reticulocyte lysate to make up a total of 4 μl of lysate within the gel shift reaction. 50,000 cpm ^{32}P-labeled probe was added to the samples in a cocktail as previously described, followed by competitor where appropriate (Hagman et al., 1991; Jacobs et al., 1994). The probe in Fig. 5 is derived from sequences in the *λ5* promoter: 5' GTTCCATGGGGCAGGTGTTCAGTTGCTCACAGACCCAGGGGCCCTCAGG GACTGGATATCAGTCAGGC, and 5' GCCTGACTGATATCCAGTCCCTGAG GGCCCCTGGGTCTGTGAGCAACTGAACCTGCCCCATGGAAC (E47-3: -272 to -266; EBF-2: -213 to -197; some intervening sequence deleted (Sigvardsson et al., 1997). The probe used in Fig. 6A, *Pax5-EBF1*, is contained in the *Pax5* promoter: 5' GGGGTGGGGGGACTCCCGGGAATCTACAGGCCAC, and 5' GTGGCCTGTAGATTCCCGGGAGTCCCCCCACCCC. Gel shift conditions for Fig. 4-6A were as previously described (Hagman et al., 1991; Jacobs et al., 1994). Double stranded oligonucleotides containing either an *mb-1* site or a *μE5* site were used as competitor DNA, as previously described (Hagman et al., 1991; Jacobs et al., 1994). Reactions were incubated for 1 hour at room temperature, then run on a 5% non-denaturing acrylamide gel containing 5% glycerol.

Pax5 promoter cloning

1.8 kilobases of the *Pax5* promoter (Genbank #AF148961) was cloned from a mouse genomic library using the Mouse GenomeWalker Kit (Clontech). Two gene specific primers containing sequence from *Pax5* exon 1A were used: (outer) 5′ GGCCAGAACCAGGGAAAGGGTGTCAGC and (inner) 5′ AAGGTGTGCAAGAGGCCCAGAGAGCAG.

Transient transfections

DNA, including the construct containing the *Pax5* promoter controlling the luciferase gene (derived from pGL3-B; Promega), was transiently transfected into NIH3T3 cells using Superfect (Qiagen). After 36 hrs, cells were lysed and analyzed for βgalactosidase and luciferase activity using the substrate, chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim), and a luciferase assay kit (Promega) respectively.

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Figure Legends

Figure 4-1.

FACS analysis of E17.5 fetal liver pro-B lymphocytes

(A) *Ebf+/-E2a*$^{+/-}$ fetal livers contain fewer pro-B cells. Anti-CD43-FITC and anti-B220-PE antibodies were used to stain fetal liver cells from E17.5 littermates. Anti-B220 is used as a pan-B cell marker, while anti-CD43 stains only pro-B cells within the B cell population. Cells shown in the FACS plots have been gated on lymphocytic cells by forward and side scatter to exclude debris and large granular cells. Numbers next to each box represent the percentage of lymphocyte gated cells shown.

(B) Graphical representation of the percentage of mutant fetal liver pro-B cells, as compared to wildtype at E17.5 dpc. In each litter, the number of B220^{+} CD43^{+} cells in a wildtype animal was set at 100%; if there were two wildtype animals, an average of the two was used as 100%.

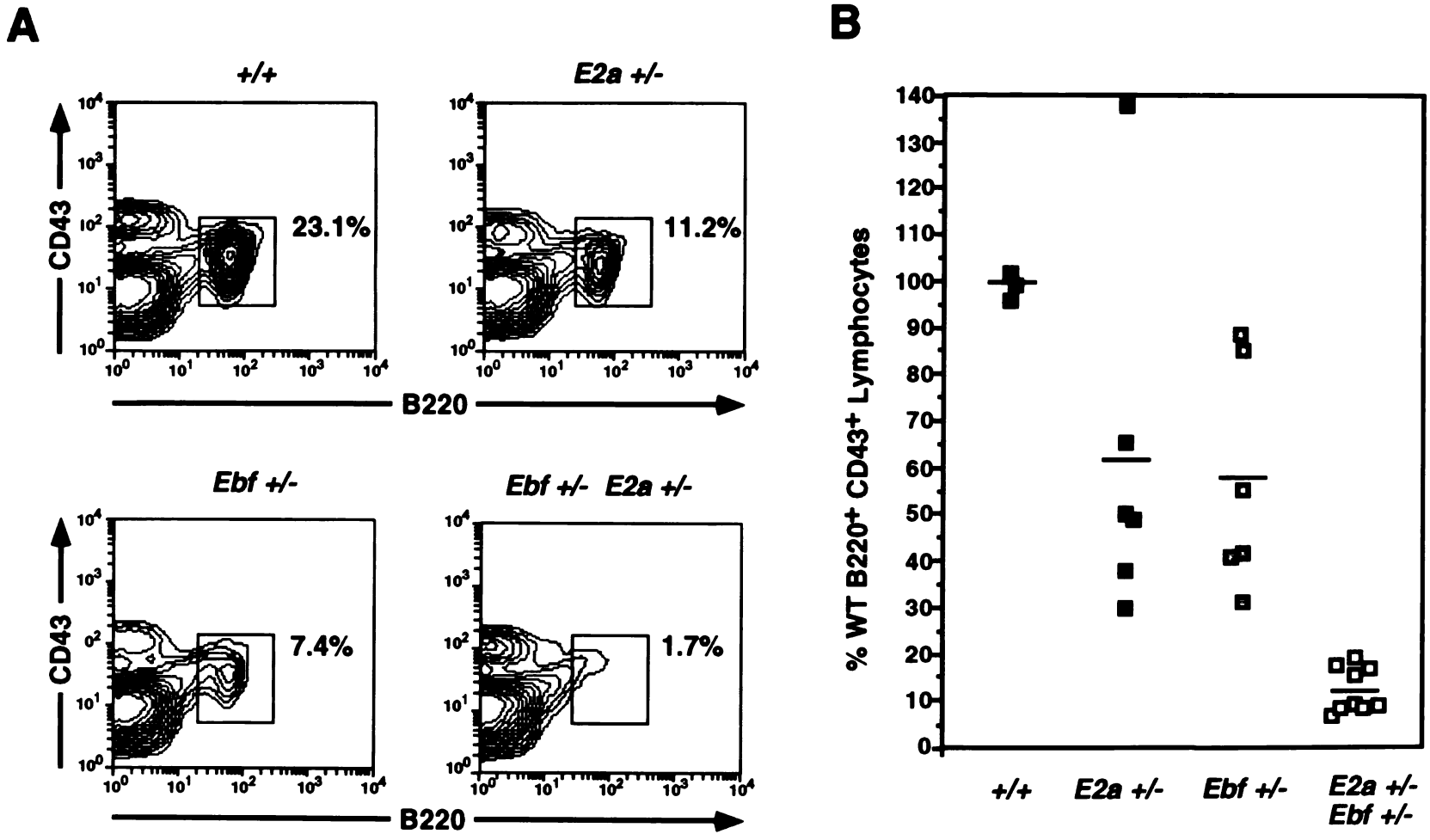

A
+/+
E2a +/-
Ebf +/-
Ebf +/- E2a +/-
CD43
B220
23.1%
11.2%
7.4%
1.7%
B
% WT B220+ CD43+ Lymphocytes
+/+
E2a +/-
Ebf +/-
E2a +/-
Ebf +/-

Figure 4-2.

Four color FACS analysis of fetal liver pro-B cell differentiation

E18.5 fetal liver cells were stained with antibodies against HSA-FITC, CD43-PE, BP-1-biotin or CD19-biotin, and B220-APC to distinguish between pro-B cell fractions.

(A) *Ebf+/-E2a+/-* fetal liver cells exhibit a defect in the fraction B to C transition. $B220^+$ $CD43^+$ gated cells are shown. $HSA^-BP\text{-}1^-$ cells represent fraction A pro-B lymphocytes, $HSA^+BP\text{-}1^-$ cells represent fraction B and $HSA^+BP\text{-}1^+$ represent fraction C. Numbers in each quadrant signify percentage of lymphocyte gated cells.

(B) *Ebf+/-E2a+/-* fetal liver cells fraction B cells express less CD19 on their surface than wildtype Fraction B cells. $B220^+$ $CD43^+$ $BP\text{-}1^-$ gated cells are displayed in histograms. The number above the marker indicates percent of fraction B cells that are $CD19^+$.

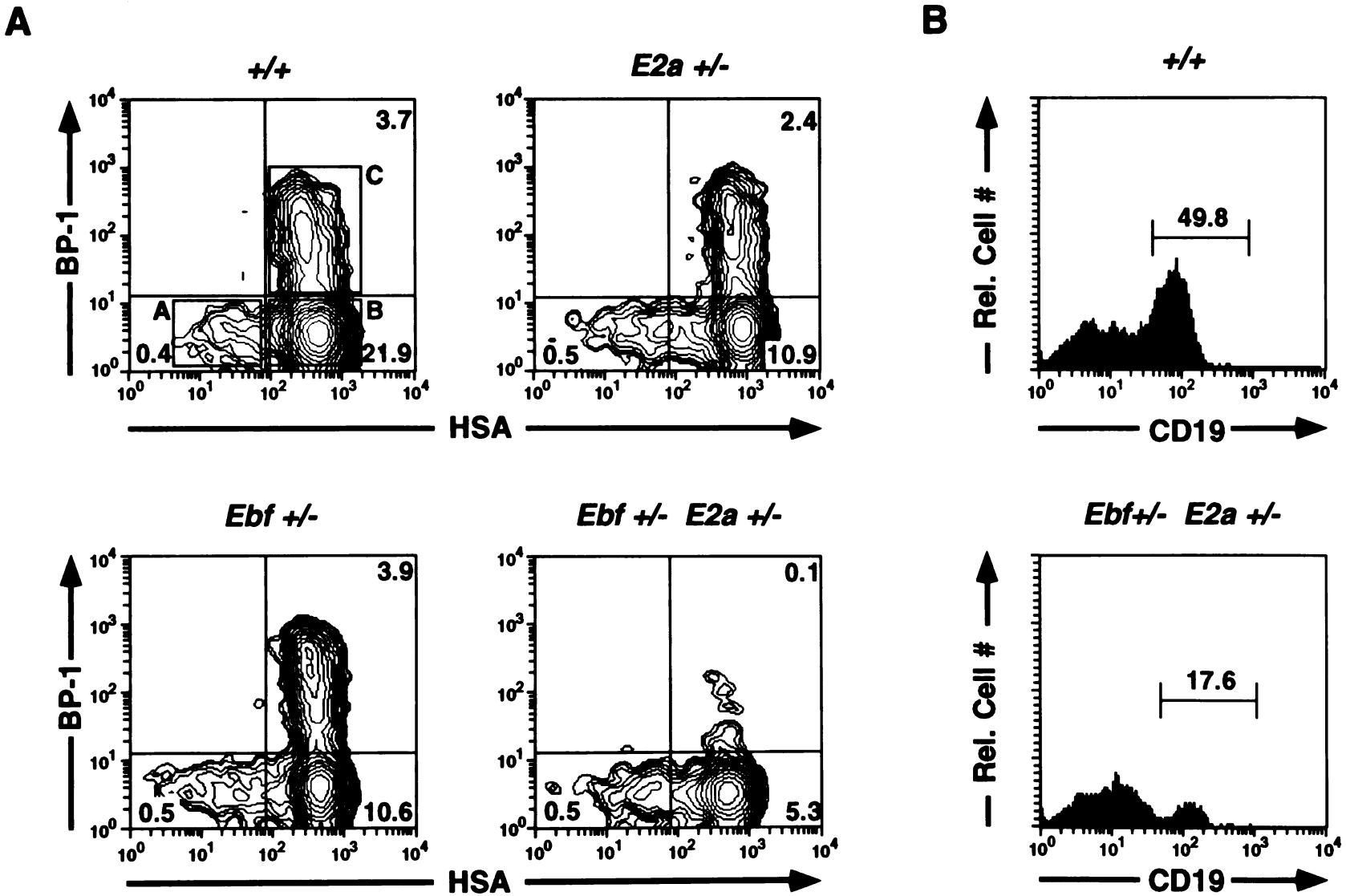

A
+/+
E2a +/-
Ebf +/-
Ebf +/- E2a +/-
BP-1
HSA
3.7
C
A
B
0.4
21.9
2.4
0.5
10.9
3.9
0.5
10.6
0.1
0.5
5.3
B
+/+
Ebf+/- E2a +/-
Rel. Cell #
CD19
49.8
17.6

Figure 4-3.

Adult *Ebf+/-E2a*$^{+/-}$ mice display a defect in B cell development

(A) Bone marrow from *Ebf+/-E2a*$^{+/-}$ adult animals exhibits a pro-B cell defect. Bone marrow cells from 4-month old littermates were stained with anti-B220-PE and anti-IgM-FITC. Early B cells are $B220^{lo}IgM^{-}$, immature B cells are $B220^{lo}IgM^{+}$ and mature recirculating cells $B220^{hi}IgM^{+}$. Numbers next to the black boxes indicate percentage of lymphocyte gated cells represented within the box.

(B) *Ebf+/-E2a*$^{+/-}$ adult mice have fewer B lymphocytes in the spleen. Spleens from 4-month old littermates were isolated and stained with anti-B220-PE antibodies. Numbers displayed above the markers represent percentage of lymphocyte gated cells.

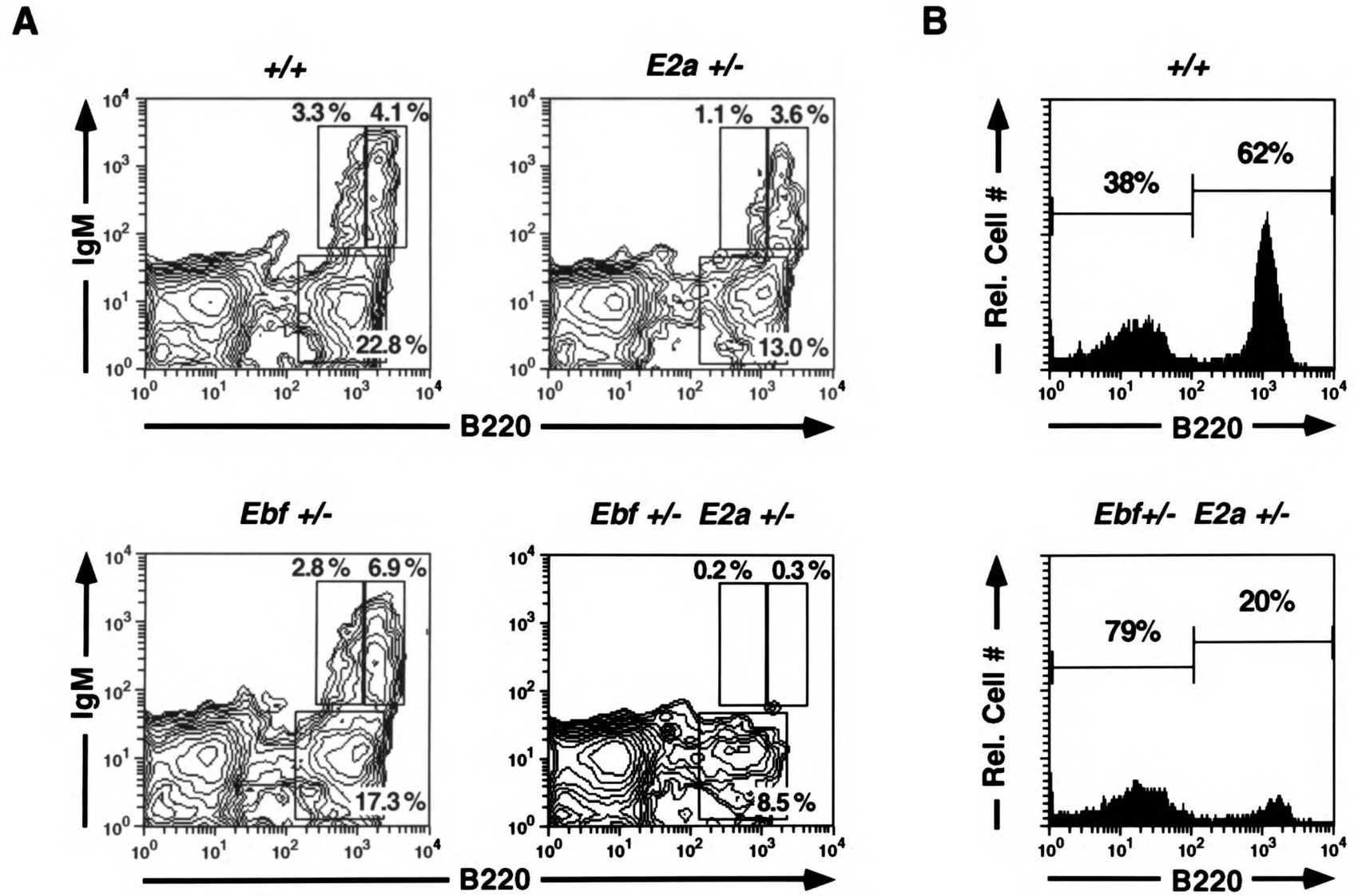

A
+/+
E2a +/-
Ebf +/-
Ebf +/- E2a +/-
IgM
B220
3.3%
4.1%
22.8%
1.1%
3.6%
13.0%
2.8%
6.9%
17.3%
0.2%
0.3%
8.5%
B
+/+
Ebf+/- E2a +/-
Rel. Cell #
38%
62%
79%
20%

Figure 4-4.

RT-PCR analysis of fetal liver pro-B cells

(A) Semi-quantitative bulk RT-PCR of wildtype E17.5 FACS sorted fraction B pro-B cells shows that titration of input cDNA results in linear amplification of *IL7R* message after 30 cycles.

(B) RT-PCR analysis of transcript levels in wildtype E17.5 pro-B lymphocyte fractions A, B and C. 30 cycle reactions are shown for EBF, E12 and E47; 20 cycles shown for actin.

(C) E15.5 fetal liver contains few fraction C cells. FACS analysis of a representative +/+ fetal liver at E15.5, stained with anti-HSA-FITC, anti-B220-PE and anti-BP-1-biotin. Only $B220^+$ cells are shown; numbers in each quadrant indicate the percentage that each fraction represents of all $B220^+$ cells.

(D) *Ebf+/-E2a+/-* fetal liver cells exhibit a significantly altered profile of gene expression. Bulk RT-PCR analysis of fetal liver cells magnetically sorted with beads coated with anti-B220 antibody. Twenty-seven, thirty and thirty-three cycles of PCR were performed for each set of primers to ensure amplification in the linear range. For *IL7R*, *λ5*, V_{preB}, *Iμ*, *B29*, and *mb-1*, 30 cycles are shown; *Rag-2* and *Pax-5* PCR reactions shown are the product of 33 cycles.

Figure 4-4. (continued)

(E) *Ebf+/-E2a+/-* fetal livers have fewer V_{preB} expressing cells. Fetal liver cells from E17.5 animals were stained with anti-B220-PE and anti-BP-1-biotin. $B220^{+}BP\text{-}1^{-}$ cells were sorted into 96 well plates and subjected to RT-PCR analysis with primers to the V_{preB} gene. Each well represents two cells.

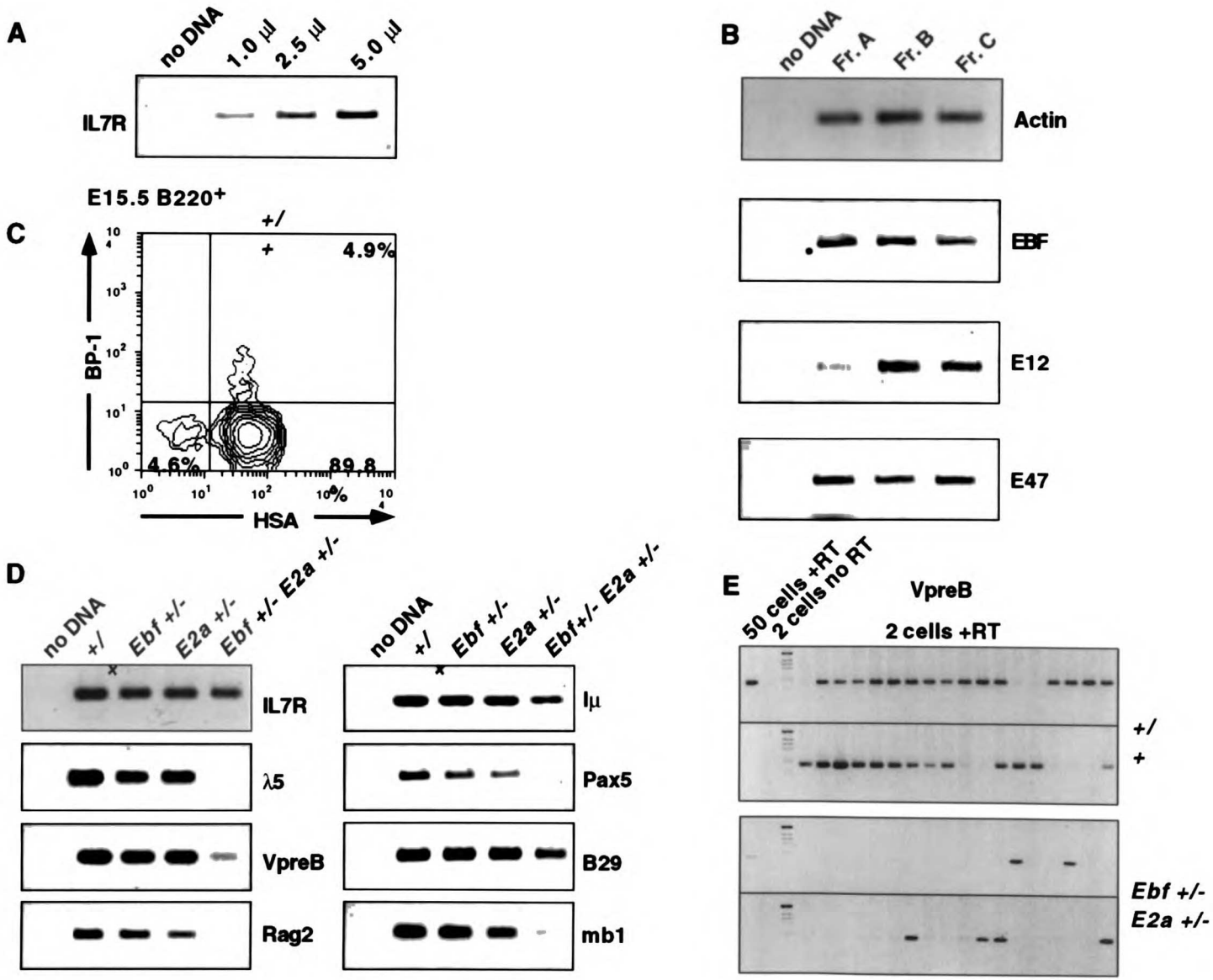

A
no DNA
1.0 µl
2.5 µl
5.0 µl
IL7R
B
no DNA
Fr. A
Fr. B
Fr. C
Actin
EBF
E12
E47
C
E15.5 B220+
+/
+
4.9%
BP-1
4.6%
89.8
HSA
D
no DNA
+/
Ebf +/-
E2a +/-
Ebf +/- E2a +/-
IL7R
λ5
VpreB
Rag2
Iμ
Pax5
B29
mb1
E
50 cells +RT
2 cells no RT
VpreB
2 cells +RT
+/
+
Ebf +/-
E2a +/-

Table 1.

Summary of RT-PCR analysis.

The symbols by each gene name represent the relative level of expression seen in RT-PCR done on *Ebf+/-E2a*$^{+/-}$ samples compared to a wildtype littermate control (++++). RT-PCR reactions that showed a difference in expression were repeated with the same samples to control for consistency, as well as with cDNA made from separate litters collected on different days. The last two rows of the table represent data from 2 or 5 cell RT-PCR analysis, an example of which is shown in Fig.4. The fractions represent the number of reactions giving a positive PCR result for V_{preB} or *Rag-2* out of total wells tested for that gene. All wells counted were positive in an *actin* RT-PCR assay.

RT-PCR Summary — Table 1

IL7-R	++++	Bcl-2	++++
Iμ	++++	Bcl-xL	++++
Mu°	++++	PU.1	++++
λ5	+	BSAP	+
VpreB	++	CD19	++
SCID	++++	E12	+++
Rag1	+	LEF-1	++
Rag2	+	Sox-4	++++
mb-1	++	TCF-4	++++
B29	+++	Fgr	++++
Cdc25A	++++	Flk	++++
Cdc25B	++++	Blk	++++
Cdc25L	++++	Ikaros-VI	++++

	+/+	*Ebf+/- E2a +/-*	Cell #
VpreB	28/36	6/36	2
Rag2	21/36	3/36	5

Figure 4-5.

EBF and E47 form a ternary complex on sequences from the λ5 promoter.

An electrophoretic mobility shift assay using a 68 bp probe derived from the λ5 promoter, that contains one E-box and one EBF site is shown. *In vitro* transcribed and translated EBF and E47 proteins were added, along with 100-fold molar excess of competitor DNA where indicated. Competitor oligonucleotides contain either an EBF site from the *mb-1* promoter (EBF) or the *μE5* E-box from the Ig heavy chain enhancer (E2A). Complexes of different mobility are identified at the side of the panel.

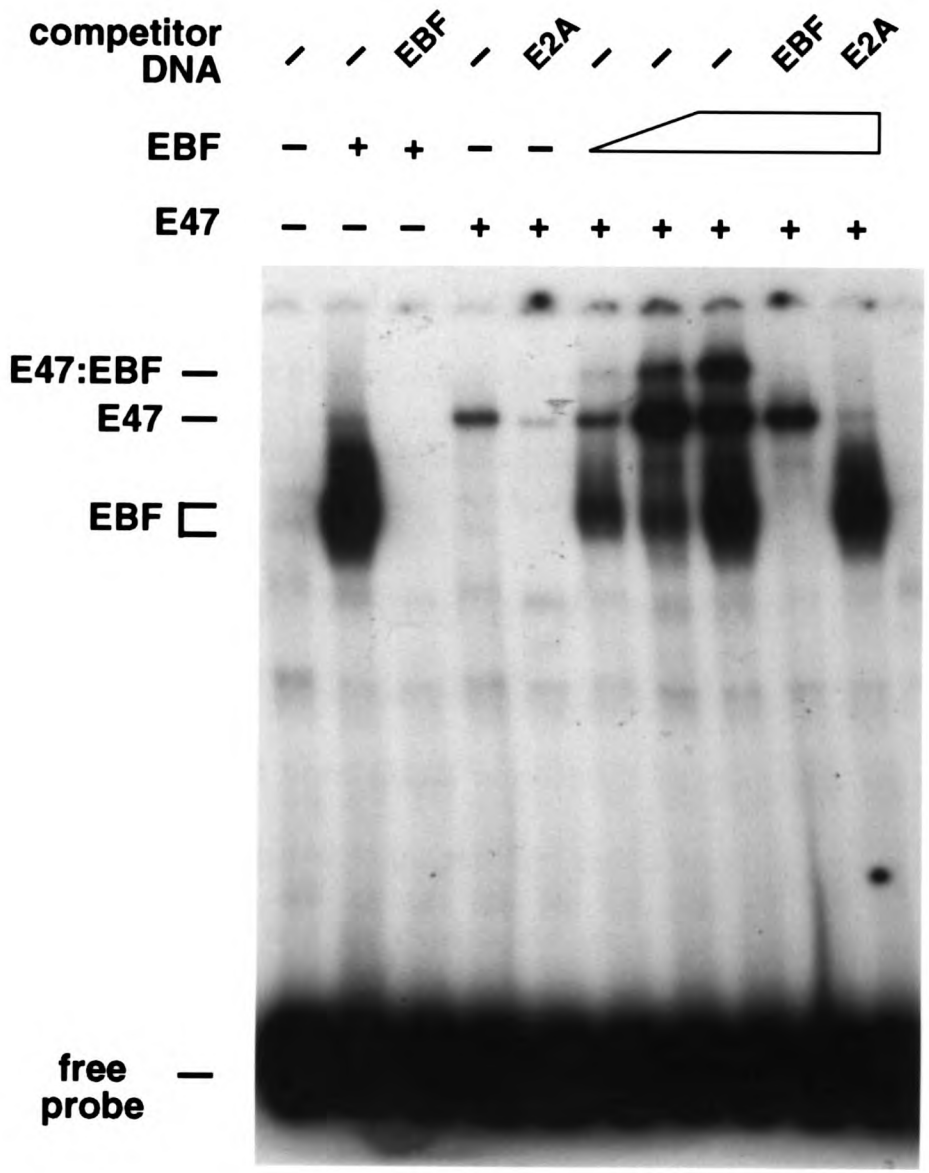
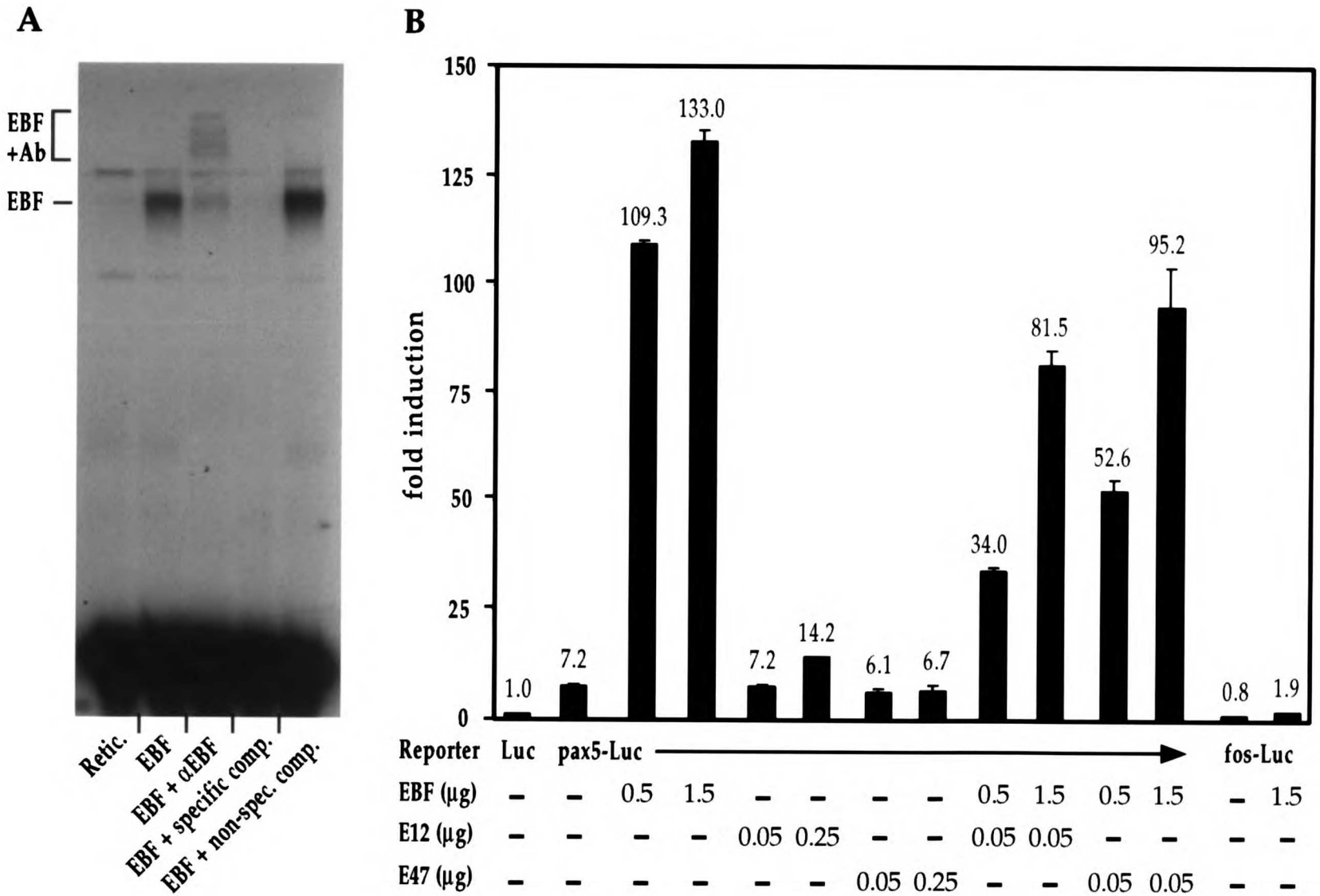

competitor DNA
–
–
EBF
–
E2A
–
–
–
EBF
E2A
EBF
–
+
+
–
–
E47
–
–
–
+
+
+
+
+
+
+
E47:EBF
E47
EBF
free probe

Figure 4-6.

EBF binds and transactivates the *Pax5* promoter.

(A) An electrophoretic mobility shift assay using a 34 bp probe derived from the 1.8 kb *Pax5* promoter (Genbank #AF148961) is shown. *In vitro* transcribed and translated EBF was added, with a 100-fold molar excess of unlabeled competitor DNA where indicated. Unlabeled competitor oligonucleotides added were either the same DNA as used for the probe (*Pax5-EBF1* - specific) or DNA containing the *μE5* E-box (non-specific). The supershifted EBF:DNA:Ab complex resulting from the addition of anti-EBF antiserum is indicated at the side of the panel.

(B) Transient transfections of NIH3T3 cells with EBF result in activation of the *Pax5* 1.8 kb promoter in a luciferase reporter construct. Fold induction represents relative light units (RLU) normalized by the expression of CMV-βgalactosidase and compared to the reporter vector alone. Each bar represents duplicate transfections, and the experiment shown is representative of three different experiments.


A
EBF
+Ab
EBF
Retic.
EBF
EBF + αEBF
EBF + specific comp.
EBF + non-spec. comp.
B
fold induction
150
125
100
75
50
25
0
1.0
7.2
109.3
133.0
7.2
14.2
6.1
6.7
34.0
81.5
52.6
95.2
0.8
1.9
Reporter Luc pax5-Luc fos-Luc
EBF (µg) – – 0.5 1.5 – – – – 0.5 1.5 0.5 1.5 – 1.5
E12 (µg) – – – – 0.05 0.25 – – 0.05 0.05 – – – –
E47 (µg) – – – – – – 0.05 0.25 – – 0.05 0.05 – –

CHAPTER 5

Identification of Genetic Targets for LEF-1 in B Cell Development

ABSTRACT

Lymphocyte enhancer factor-1 (LEF-1) is a member of the LEF/TCF family of high mobility group (HMG) DNA binding proteins. LEF-1 has been shown to play a role in organogenesis and the development of the T lymphocyte lineage. LEF-1 is also expressed in B lymphocytes, however, its role in B cell differentiation is unknown. Here we examine the function of LEF-1 as a transcriptional regulator of lymphocyte development by analyzing the expression of candidate target genes in wildtype or *Lef*$^{-/-}$ B cells. We show that primary *Lef*$^{-/-}$ B lymphocytes exhibit elevated levels of terminal deoxynucleotidyl transferase (*TdT*), whose gene product contributes to antibody diversity. We also find altered expression of the *fas*, *c-myc* and *N-myc* genes, which are known to participate in cellular survival and proliferation. Taken together, our observations support a regulatory role for LEF-1 in promoting survival and proliferation in the developing immune system.

Introduction

The complex process of lymphocyte differentiation requires both intrinsic and extrinsic components. Each developing cell follows a genetic program that specifies stage and lineage specific gene expression. These lymphocytes are also dependent on direct contact and signals from their surrounding environment, the fetal liver, thymus or bone marrow. External cues provided by stromal cells are integrated into the B cell developmental program at many different levels, including transcriptional regulation.

LEF-1 is a member of the LEF/TCF family of transcription factors, that are expressed in sites of organogenesis, as well as in B and T lymphocytes (Oosterwegel et al., 1993; Travis et al., 1991; van Genderen et al., 1994). Other family members include TCF-1 which plays an overlapping role with LEF-1 in T cells, and TCF-4, also expressed in developing B lymphocytes (Korinek et al., 1998; Okamura et al., 1998; Verbeek et al., 1995). During B cell differentiation, LEF-1 is present in pro-B cells (Fractions B and C), but expression is downregulated during maturation (Tannishtha Reya, Eric Devaney, Rudolf Grosschedl, unpublished observations). The fetal livers of *Lef*$^{-/-}$ mice contain significantly fewer B cells than wildtype mice, although the remaining cells are able to differentiate to maturity (T. Reya, R. Grosschedl, unpublished observations). These data suggest a role for LEF-1 specifically in the survival or proliferation, rather than the differentiation, of developing B lymphocytes.

The LEF/TCF proteins have three functional domains (see Fig 1-4B): a beta-catenin interaction domain, a context dependent activation domain and the HMG box which binds DNA (Behrens et al., 1996; Giese and Grosschedl, 1993). LEF-1 is known to act, by binding and sharply bending DNA, as an architectural protein in the assembly of a higher order nucleoprotein complex on the T cell receptor alpha enhancer (Giese et al., 1992; Giese et al., 1995). In this context, LEF-1 depends on the presence of other factors in the complex, such as ALY, to mediate transcriptional activation of the TCRα gene (Bruhn et al., 1997). In the activation of other known target genes, such as *siamois*, LEF/TCF family members interact with β -catenin, an intermediate of the Wnt/Wingless signaling pathway (see Fig. 1-4A) (Fan et al., 1998). Thus, the function of LEF-1 in activating some target genes during lymphocyte differentiation may be directed by external signals.

The Wnt/Wingless signaling cascade is a highly conserved pathway that participates in diverse developmental processes (Cadigan and Nusse, 1997). The Wnt proteins are secreted and send signals by binding to the receptor, frizzled and its family members. Binding of frizzled leads to the inhibition of GSK-3β kinase, a regulator of beta-catenin stability, resulting in stabilization and accumulation of beta-catenin. Beta-catenin is imported into the nucleus in a Wnt dependent manner (Fagotto et al., 1998; Hsu et al., 1998). In the nucleus, β-catenin can form a complex with LEF/TCF family members to regulate transcription (Eastman and Grosschedl, 1999). The role of the Wnt pathway in

determining cell fate has been extensively characterized in *Drosophila* embryogenesis (Wodarz and Nusse, 1998). However, the function of this pathway in the context of lymphocyte development is not well understood, although some of the proteins involved in the pathway are expressed in lymphocytes or stromal cells (Austin et al., 1997; Van Den Berg et al., 1998). In other mammalian cell types, the Wnt pathway has been implicated in survival and proliferation (Bradley and Brown, 1995; He et al., 1998; Morin et al., 1997; Tetsu and McCormick, 1999). Identifying target genes of LEF-1, a downstream effector of the Wnt pathway, in the immune system will add to our understanding of the complex interactions that occur during lymphocyte differentiation. Here we show that LEF-1, directly or indirectly, regulates genes involved in the generation of antibody diversity, and in the survival and proliferation of B lymphocytes.

Results

Lef1 expression is temporally regulated

In order to determine what genes might be candidates for regulation by LEF-1, we studied the expression of *Lef1* itself in different wildtype B cell populations (Fig. 5-1). We first isolated developing B cells from the bone marrow or spleen of an adult mouse by staining with antibodies against the pan-B cell marker, B220, and immunoglobulin. These cells were sorted by flow cytometry. In the bone marrow, $B220^{lo}IgM^-$ cells represent early B cells, and $B220^{lo}IgM^+$ cells

represent maturing B cells; $B220^{hi}IgM^{+}$recirculating B cells were excluded. $B220^{+}IgM^{+}$ lymphocytes from the adult spleen were also collected. RNA was extracted from the sorted cells and subjected to semi-quantitative RT-PCR analysis (Fig. 5-1A). cDNA levels were normalized based on amplification of actin cDNA. *Lef1* expression is found in the early B cell compartment, but not in later stages of B cell development, nor in peripheral B lymphocytes. Thus, target genes of LEF-1 are likely to be regulated during pro- or pre-B cell differentiation.

Lef$^{-/-}$ mice exhibit a high rate of neonatal mortality, and many of our experiments are performed using fetal tissue. Many genes are differentially expressed and play distinct roles in fetal and adult tissues, such as the terminal deoxynucleotidyl transferase (*TdT*) gene in the lymphoid lineage (Gilfillan et al., 1993; Komori et al., 1993). Furthermore, B lymphopoiesis occurs in different organs, thus in different stromal environments, in the embryo and the adult. We therefore tested the expression of *Lef1* in adult versus fetal B lymphopoiesis. We isolated $B220^{+}IgM^{-}$ lymphocytes from bone marrow and E18.5 fetal liver that should represent roughly equivalent populations. The sorted cells were used for semi-quantitative RT-PCR analysis (Fig. 5-1B). cDNA was normalized based on the relative amplification of actin transcripts. *Lef1* expression is markedly higher in fetal liver B cells than adult bone marrow B cells. Conversely, the *TdT* gene is expressed at high levels in adult B lymphocytes but is barely detectable in fetal liver B lymphocytes, consistent with previously published observations (Li et al., 1993). We conclude from these data that *Lef1* is expressed in a temporally

regulated manner. Thus regulation by LEF-1 is most likely to be important in the fetal liver early B cell compartment.

Lef$^{-/-}$ fetal liver pro-B cells exhibit an altered gene expression profile

To identify potential targets of LEF-1 that might affect survival or differentiation, we examined the gene expression profile of fetal liver pro-B cells using the candidate gene approach. E18.5 fetal liver cells were stained with antibodies against the surface antigens, B220 and BP-1. RNA was isolated from B220^{+}BP-1^{-} cells that were sorted by fluorescence activated cell sorting (FACS). The RNA samples were reverse transcribed and the resulting cDNA subjected to semi-quantitative amplification by gene specific primers (Fig. 5-2). We found that the expression of several genes was increased in *Lef$^{-/-}$* pro-B cells. The related gene, *Tcf4*, was expressed at normal levels *in vivo* in all animals. Expression of *TdT* appeared to be increased in *Lef* deficient fetal liver pro-B cells. The TdT protein is expressed differentially in fetal liver and bone marrow lymphocyte development, where it increases B or T cell receptor diversity by adding random nucleotides, termed N-region additions, during recombination (Gilfillan et al., 1993; Komori et al., 1993; Li et al., 1993). Since LEF-1 is expressed at higher levels in fetal liver than bone marrow pro-B cells, it is possible that LEF-1 may be involved in the repression of *TdT*. Recent studies have shown that LEF-1 can mediate transcription repression through the family of Groucho repressors(Levanon et al., 1998). However, homologous disruption of the *TdT*

gene has no effect on B lymphocyte development; thus, the increase in *TdT* expression in *Lef/-* pro-B cells is unlikely to contribute to the decrease in the B cell compartment observed in the *Lef/-* mice (Gilfillan et al., 1993; Komori et al., 1993).

Fas transcripts were also significantly upregulated in *Lef* deficient mice. This observation is consistent with the increased cell surface expression of Fas seen by flow cytometric analysis (T. Reya and R. Grosschedl, unpublished observations). The Fas receptor can trigger apoptosis in lymphocytes upon binding of its ligand, FasL (Cohen and Eisenberg, 1991; Nagata and Suda, 1995). *FasL* expression was not reproducibly altered in *Lef* deficient lymphocytes (data not shown). The expression of the proto-oncogene, *c-myc*, was also increased in the *Lef* deficient fetal liver, compared to the wildtype. Interestingly, the expression of *N-myc*, a related gene, is downregulated in *Lef/-* pro-B cells. The expression of other survival or apoptosis inducing factors were tested such as *Bcl-2*, *Bcl-x*, *Bax* and *p53*, however none of these appeared to be affected in the *Lef/-* samples at the RNA level (data not shown). We conclude from these data that LEF-1 is required for the normal expression of multiple genes, which are involved in recombination, survival and proliferation. It remains unclear whether LEF-1 directly regulates these genes in the lymphocyte lineage.

N-myc and Tcf4 respond differentially to IL7 in Lef/- pro-B lymphocytes

N-myc and *c-myc* are early response genes, rapidly induced by IL7 (Morrow et al., 1992). Their differential regulation in *Lef/-* pro-B cells suggests

that pro-B cells without LEF-1 may not respond normally to extracellular signals. However, *Lef/-* pro-B cells cultured *ex vivo* in IL7 appeared to proliferate normally (T. Reya, R. Grosschedl, unpublished observations). In order to test the transcriptional response of *Lef/-* pro-B cells to IL7 withdrawal, primary fetal liver pro-B cells were cultured *ex vivo* in the presence of IL7, an early B cell growth and survival factor, for two weeks. The cells were then washed and cultured in media without IL7 for six hours. IL7 was added back to the media for a maximum of two hours. Cells were harvested for RNA preparation after two weeks in IL7, six hours after IL7 withdrawal, and one half and two hours after the subsequent addition of IL7. The RNA was reverse transcribed and subjected to semi-quantitative PCR analysis (Fig. 5-3). Many genes, such as *Fas*, *FasL* and *c-myc* were expressed in cultured *Lef/-* pro-B cells indistinguishably from wildtype cells (data not shown). Interestingly, the LEF family member, *Tcf4*, was strongly upregulated in *Lef/-* pro-B cells in response to IL7 withdrawal, suggesting that LEF-1 may feed back negatively upon the expression of *Tcf4*, which is normally upregulated later in B cell development (M. O'Riordan, T. Reya and R. Grosschedl, unpublished observations). The specific induction of *Tcf4* in *Lef/-* pro-B cells upon IL7 withdrawal could indicate a sensor in the cell, responsive to the presence of LEF or TCF4, which is activated under conditions of stress. *N-myc* transcription was also increased in *Lef/-* pro-B cells to a greater extent than the wildtype pro-B cells. Thus, LEF-1 affects the basal and inducible state of *N-*

myc transcription, and the inducible state of *Tcf4* transcription, in response to the B cell growth factor, IL7.

Differential regulation of fas, c-myc and N-myc in the fetal liver and thymus of Lef/- mice

Since some of the genes affected in *Lef/-* pro-B cells are also expressed in T cells, we examined the gene expression profile in *Lef/-* developing T cells. *Lef/-* mice do not exhibit any apparent perturbation of T cell differentiation (Okamura et al., 1998). Different populations of developing T lymphocytes appear as normal, in approximately normal numbers. T cells express high levels of the LEF family member, TCF-1, which allows T cell precursors to survive and mature (Schilham and Clevers, 1998; Verbeek et al., 1995). We examined gene expression in the fetal thymus to determine if the changes we observed in the fetal liver were occurring in the general lymphocyte population. Whole fetal thymi from wildtype and *Lef/-* animals were homogenized, and RNA harvested. Although the fetal thymus largely consists of developing T cells, stromal cells were also contained in the preparation. The RNA was reverse transcribed into cDNA and subjected to semi-quantitative RT-PCR analysis (Fig. 5-4). The pan-T cell marker, Thy-1, was used to normalize the cDNA samples. The results differed significantly from those of the *Lef/-* fetal liver analysis. *Fas* expression in the *Lef/-* fetal thymus was similar to the wildtype, which expressed higher levels of *Fas* than those seen in B lymphocytes. Developing T cells undergo a rigorous selection that results in the death of large numbers of differentiating cells in the

thymus (Kishimoto et al., 1998; Nishimura et al., 1995; Ogasawara et al., 1995; Van Parijs et al., 1998; Watanabe-Fukunaga et al., 1992). Since Fas is normally expressed in this tissue, it is possible that *Fas* expression cannot be upregulated any further. The levels of *c-myc* were also unchanged. Surprisingly, the levels of *N-myc* were different between the *Lef*$^{-/-}$ fetal thymus and the wildtype, but in a manner opposite to that observed in fetal liver pro-B cells. Therefore, we conclude that LEF-1 does not regulate genes in the developing thymus in a similar manner to the fetal liver, possibly due to the overlapping function of TCF-1.

Discussion

Members of the LEF/TCF family are expressed during lymphoid development, and are known to be important in T cell differentiation (Korinek et al., 1998; Okamura et al., 1998; Verbeek et al., 1995). Here we show that in *Lef*$^{-/-}$ fetal liver pro-B cells, the genes *TdT*, *fas*, *c-myc* and *N-myc* are differentially expressed. We further observe differential upregulation of the LEF/TCF family member, *Tcf4*, and *N-myc*, under IL7 withdrawal conditions. Finally, expression analysis of these genes in *Lef*$^{-/-}$ fetal thymus suggests that the ability of LEF-1 to regulate downstream target genes depends on the cellular context.

LEF-1 itself does not appear to play a critical role in the maturation of B or T lymphocytes, although it may do so redundantly with other family members, such as TCF-1 or TCF-4. Rather, LEF-1 may be important in reading signals that

determine whether B cell survive and proliferate. This hypothesis is consistent with the known participation of LEF/TCF family members in the Wnt signaling pathway (Aoki et al., 1999; Behrens et al., 1996; Brunner et al., 1997; Cavallo et al., 1997; Eastman and Grosschedl, 1999; Galceran et al., 1999; Hsu et al., 1998; McKendry et al., 1997; van de Wetering et al., 1997). Clearly LEF-1 is not absolutely required for the survival of all lymphocytes, thus suggesting that LEF-1 may be relaying specific developmental signals, unlike IL7 which stimulates the growth and survival of the entire pro-B cell compartment.

The critical function of LEF-1 in developing lymphocytes is unclear. Although some evidence directly links LEF/TCF family members to genes involved in proliferation, no direct link has been established that identifies a direct target for LEF-1 in B cell differentiation (Ahmed et al., 1998; Austin et al., 1997; Bradley and Brown, 1995; Eastman and Grosschedl, 1999; He et al., 1998; Morin et al., 1997; Tetsu and McCormick, 1999). Our data showing that *Fas* and *c-myc* are upregulated in *Lef*$^{-/-}$ pro-B cells suggest that the products of these genes are normally directly or indirectly repressed by LEF-1. Upregulation of *Fas* in *Lef*$^{-/-}$ pro-B cells may contribute to the decreased ability of these cells to survive in vivo, resulting in a smaller B cell compartment. Overexpression of *c-myc* induces cell death in fibroblasts under conditions of growth factor withdrawal (Evan et al., 1992). Furthermore, c-myc has been associated with fas-mediated cell death in lymphocytes (Hueber et al., 1997). Recently, a study of the Wnt signaling pathway in a colon carcinoma cell line revealed the presence of two functional

LEF/TCF sites in the *c-myc* promoter (He et al., 1998). Thus, the increase in *c-myc* transcripts observed in primary *Lef* deficient B cells may be a direct consequence of the absence of LEF-1. Until now, there has been no evidence linking LEF-1 and *c-myc* regulation in the lymphocyte lineage. LEF-1 might be also required for a critical function that is not specifically related to survival or proliferation. The failure of that function may predispose differentiating cells to apoptosis. Clearly, an *in vitro* system that is more amenable to manipulation will allow some of these possibilities to be tested.

The role of LEF-1 as a potential mediator of Wnt signaling in lymphocyte development is of great interest, as it reflects a point of interaction between the lymphocyte and its environment. The response to extrinsic signals, such as IL7 or stem cell factor (SCF), is known to have an important effect on B cell development (Ashany et al., 1995; Billips et al., 1992; Gunji et al., 1991; Hirayama et al., 1992; Kunisada et al., 1992; Melchers et al., 1994; Rico-Vargas et al., 1994; Widmer et al., 1990). It is intriguing to speculate that in lymphocytes, as it has been shown in other cell types, signaling through the Wnt pathway may provide specific positional information. Very little is known about specific positional requirements for B cell development in the fetal liver or bone marrow, however, the thymus has a distinct architecture that is known to be important in the education and maturation of T cells. Stromal cells of the fetal liver and bone marrow are heterogeneous in character, creating greater complexity in an already complex system. Evidence for a link between Wnt signaling and the

requirement for LEF-1 in lymphocyte survival and differentiation would add a physical dimension to B cell development that has been, as yet, unexplored, but is well known in organogenesis. In the case of tooth development, where LEF-1 is absolutely required, signals are exchanged back and forth between the mesenchymal layer and the epithelium. LEF-1 appears to be required in both tissues at different developmental stages. Wnt signaling is also implicated in organogenesis, in a similar fashion. Both LEF-1 and several Wnt family members are expressed in fetal liver stroma (M. O'Riordan, T. Reya and R. Grosschedl, unpublished observations; Austin et al., 1997; Van Den Berg et al., 1998). Thus, it is possible that LEF and the Wnt signaling pathway play a similar role in the intricate process of lymphocyte development as they do in organogenesis. Further studies will be required to link LEF-1 and Wnt signaling to specific events in lymphocyte differentiation.

Materials & Methods

RT-PCR

Fetal liver cells were FACS sorted (FACStar; Becton Dickinson) into eppendorf tubes containing 10 μg yeast tRNA. Each sample shown represents one fetal liver; no samples were pooled. RNA for bulk RT-PCR was purified with Trizol (Gibco BRL). The samples were treated with RQ1 DNase (Promega) before inactivation by phenol/chloroform extraction and ethanol precipitation. The fetal liver bulk RNA samples were then reverse transcribed with MMLV-RT

(Gibco BRL). The cDNA reactions were brought up to a final volume of 100 µl and 5 µl used in an RT-PCR reaction. All bulk RT-PCR reactions were standardized by levels of β-actin or by levels of Thy-1 as indicated in the figure legends. Standard RT-PCR conditions were as follows: 94° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec, using 0.1 µl of Taq polymerase (Boehringer Mannheim) per reaction for the number of cycles indicated in the figure legend. Aliquots were withdrawn at 27, 30 and 33 cycles to determine the minimum amplification necessary for detection. Primers used for bulk RT-PCR reactions were designed using GeneWorks software, and are as follows: actin and TdT (see RT-PCR, Materials & Methods, Chapter 3), Lef-1 and Tcf-4 (see RT-PCR, Materials & Methods, Chapter 4), Fas (Genbank #M83649) (1) 5'CACACTCTGC GATGAATAGC, (2) 5' ATTGGTACCAGCACAGGAGC; c-myc (Genbank #X01023) (1) 5' TCCTGTACCTCGTCCGATTC, (2) 5' TTCCAAGACGTTGTGT GTCC; N-myc (Genbank #M12731) (1) 5' CCTCCTCTAACAACAAGGCG, (2) 5' CCTTCTCGTTCTTCACCAGC; Thy-1 (Genbank #M10246) (1) 5' GAACTCTTGGCACCATGAAC, (2) 5' CCACACTTGACCAGCTTGTC. All PCR reactions were run on a 2% agarose gel with ethidium bromide and visualized by UV transillumination. Images were captured with a gel documentation system (Alpha Innotech).

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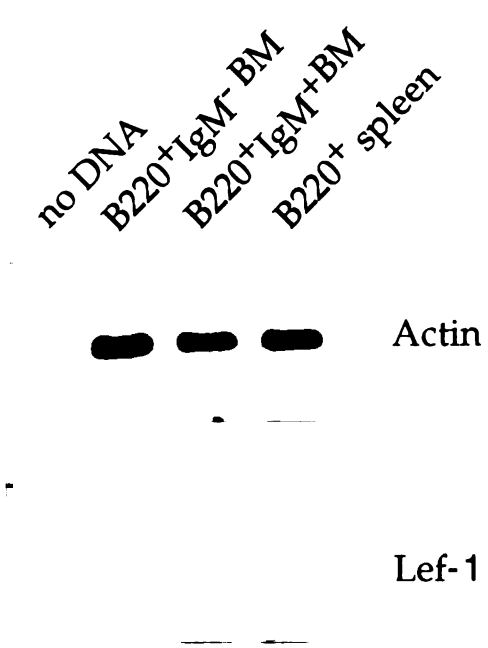
Figure 5-1.

Expression analysis of *Lef1* in B lymphocytes.

(A) Semi-quantitative RT-PCR analysis of FACS sorted B lymphocytes from adult bone marrow and spleen is shown. PCR products represent 20 cycles of amplification for *actin*, and 30 for *Lef1*.

(B) Semi-quantitative RT-PCR analysis of FACS sorted $B220^{+}IgM^{-}$ adult bone marrow and fetal liver B cells is shown. PCR products represent 20 cycles of amplication for *actin*, 30 for *Lef1* and 33 for *TdT*.

A


no DNA
B220+IgM- BM
B220+IgM+BM
B220+ spleen
Actin
Lef-1

B

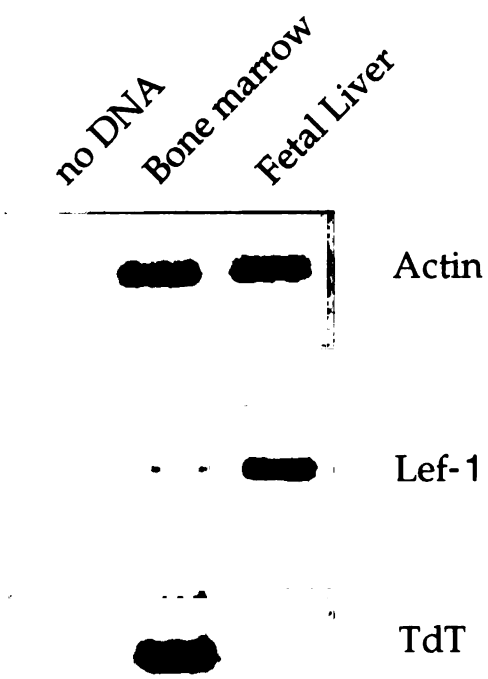

no DNA
Bone marrow
Fetal Liver
Actin
Lef-1
TdT

Figure 5-2.

Expression analysis of *Lef*$^{+/+}$ and *Lef*$^{-/-}$ fetal liver pro-B cells. E18.5 fetal liver cells were stained with fluorescent antibodies against B220 and BP-1. B220^{+}BP-1^{-} cells were collected, and lysed for RNA. RT-PCR reactions shown represent 30 cycles of amplification for all genes, except actin which was amplified for 20 cycles.

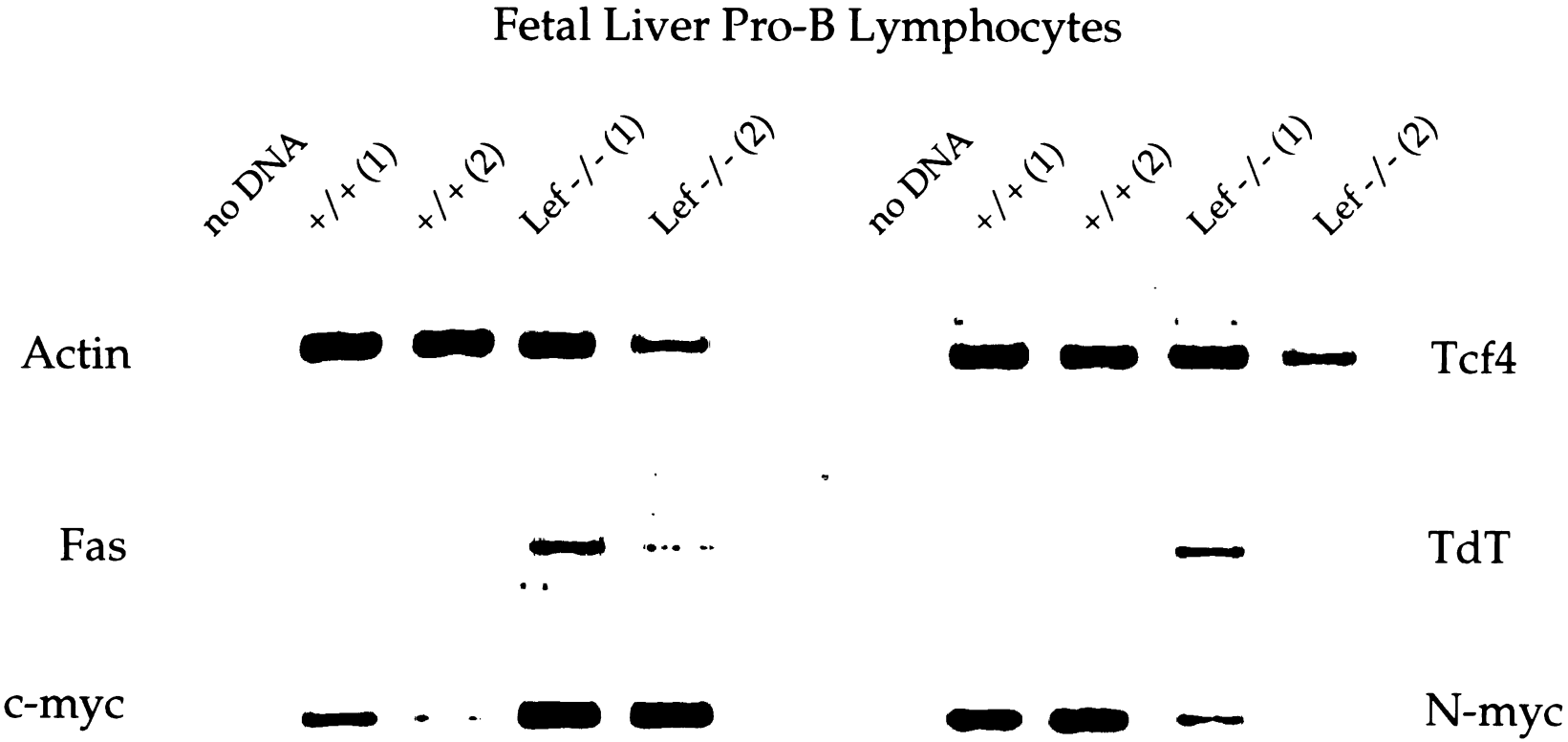

Fetal Liver Pro-B Lymphocytes
no DNA
+/+ (1)
+/+ (2)
Lef -/- (1)
Lef -/- (2)
no DNA
+/+ (1)
+/+ (2)
Lef -/- (1)
Lef -/- (2)
Actin
Tcf4
Fas
TdT
c-myc
N-myc

Figure 5-3.

Differential gene regulation in *Lef*$^{+/+}$ and *Lef*$^{-/-}$ pro-B cells upon IL7 withdrawal. B220^{+}BP-1^{-} E18.5 fetal liver cells were collected by FACS and cultured with 100U/ml recombinant IL7 for two weeks (+IL7 14 days). Cultures were extensively washed and resuspended in media without IL7 for six hours (-IL7 6 hours). rIL7 was added back to 100U/ml and cells were collected at 30 minutes and two hours subsequently (+IL7 0.5 hours; +IL7 2 hours). RNA was extracted from the samples and used in semi-quantitative RT-PCR analysis. cDNA was normalized based on relative levels of actin transcripts. PCR reactions shown represent 30 cycles of amplification for all genes, except actin which was amplified for 20 cycles.

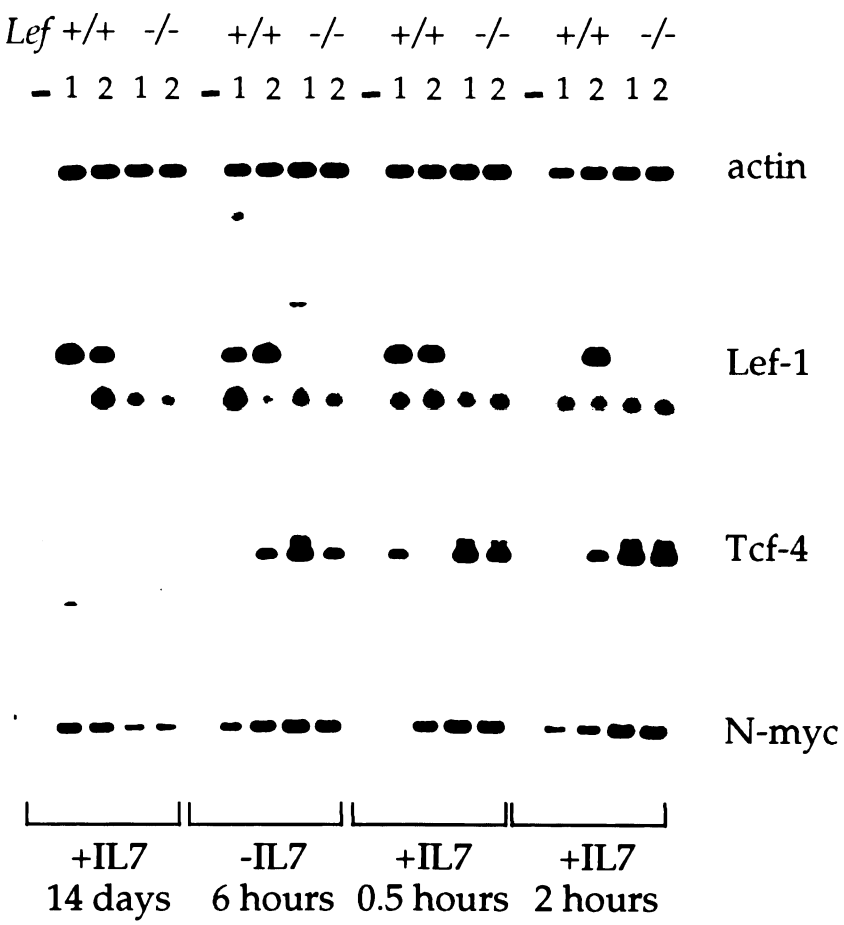
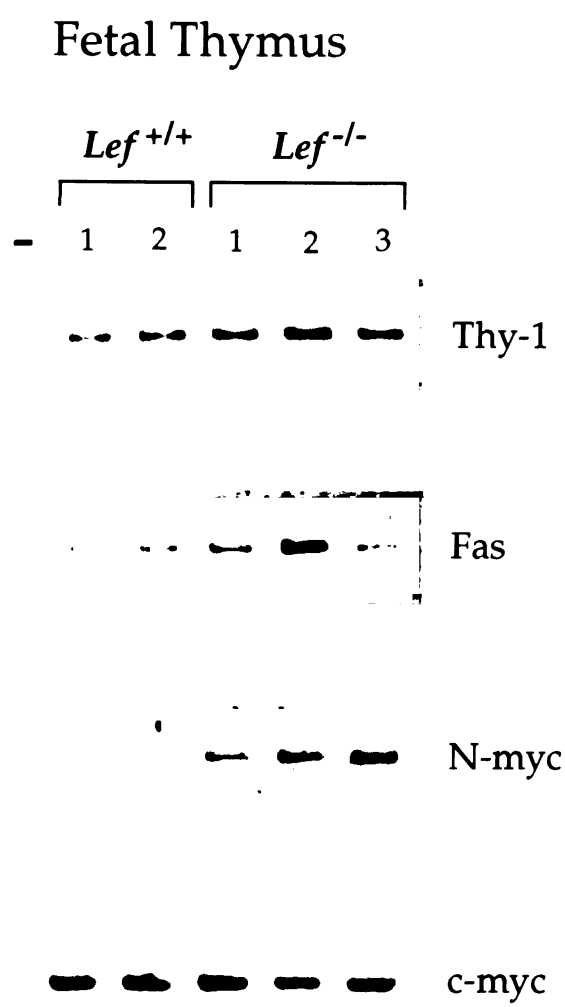

Lef +/+ -/- +/+ -/- +/+ -/- +/+ -/-
- 1 2 1 2 - 1 2 1 2 - 1 2 1 2 - 1 2 1 2
actin
Lef-1
Tcf-4
N-myc
+IL7
14 days
-IL7
6 hours
+IL7
0.5 hours
+IL7
2 hours

Figure 5-4.

Analysis of target gene expression in fetal thymus. Whole E18.5 fetal thymi were homogenized and used for RNA extraction. Three *Lef* homozygous mutants and two wildtype littermates are shown. RNA was subjected to semi-quantitative RT-PCR analysis. cDNA was normalized based on relative levels of Thy-1 transcripts. PCR reactions represent 30 cycles of amplification, except for Thy-1 which was amplified for 25 cycles.


Fetal Thymus
Lef +/+
Lef -/-
1
2
1
2
3
Thy-1
Fas
N-myc
c-myc

CHAPTER 6

Conclusion

The development of the immune system is an intriguing and complex process that still poses many questions to immunologists. In recent years, much progress has been made at the molecular level that provides a detailed, if fragmented picture, of the cellular pathways involved. Gene targeting technology has also provided valuable information about the contribution of specific genes to hematopoietic development. This thesis has focused on establishing a hierarchy of transcription factors that specifically regulate, within the hematopoietic system, B lymphocyte differentiation. We have made direct connections between factors implicated in B cell development. However, much of the network of interactions surrounding these regulatory proteins is still obscure. Here we summarize our conclusions, as well as advances made in the field, and indicate some possible directions for the future.

There is compelling evidence that EBF is an important regulator of B lymphocyte development (Lin and Grosschedl, 1995; Sigvardsson et al., 1997, see Chapter 4). This has been clearly demonstrated in Ebf deficient mice, which have no mature B cells (Lin and Grosschedl, 1995). EBF and its family members have recently been implicated in neural differentiation as well, in C. elegans, Xenopus and mouse (Dubois et al., 1998; Garel et al., 1997; Prasad et al., 1998; Wang et al., 1997). Most of the research in the area of development has identified specific developmental processes for which EBF, and related genes, are required. We have chosen to pursue genetic targets of EBF, and the interactions that result in

target gene activation in the hope of understanding more about the function of EBF in differentiation.

We have shown that EBF and collier, its Drosophila homolog, function equally well in mammalian systems, indicating that amino acid sequences that have been evolutionarily conserved likely perform critical regulatory functions (Chapter 2). One of these sequences, a helix-loop-helix domain, appears to contribute to transcriptional activation in a B cell line. This domain may mediate interaction with other regulatory factors, such as Roaz, a zinc finger protein that was cloned by virtue of its ability to bind EBF (Olf-1) (Tsai and Reed, 1997). Interestingly, Roaz can either repress or activate transcription by EBF, depending on the context of the DNA sequence. However, Roaz is unlikely to affect transcription by EBF in B lymphocytes since it is expressed poorly in that tissue. It is likely that tissue specific regulators of the activity of EBF provide flexibility and adaptability to allow EBF and its network of associated proteins to specify differentiation of diverse tissue types. Thus, a protein like EBF that acts to regulate the differentiation of motor neurons in worms may similarly be required for development of B lymphocytes. A protein that directly interacts with EBF in B cells has not yet been found. Identification of such a protein would shed light on the events that occur during a critical stage of B cell commitment and differentiation.

EBF binding sites have been identified in the promoters of several different B cell specific genes (Akerblad et al., 1999; Hagman et al., 1991;

Sigvardsson et al., 1996, see Chapter 3). However, the expression of these genes, such as Igκ, did not seem consistent with expression of EBF during B cell differentiation. Since EBF has a clear role in early B cell development, we set out to identify in vivo targets for which EBF was required. Previous experiments in the laboratory has indicated that EBF alone was not sufficient to induce changes in heterologous cell types. The transcription factor, E2A, was a good candidate for a collaborative partner in transcriptional regulation, due to its clear requirement at a similar stage of B cell development. E2A and related bHLH proteins have been implicated in myogenesis and neurogenesis, suggesting that the function of these activators is to regulate cell type specific differentiation. Expression of EBF and E2A in an immature hematopoietic cell line identified two endogenous targets, the immunoglobulin surrogate light chains, λ5 and V_{preB} (Chapter 3). The expression of these target genes during development is consistent with regulation by EBF and E2A. Furthermore, these genes are truly B cell specific, so that the intersection of EBF, also expressed in neurons, and E2A, expressed ubiquitously, resulted not only in the upregulation of genes specific to B lymphocytes, but genes that have no homolog in less complex organisms, such as Drosophila. However, many B cell specific genes were not activated upon expression of EBF and E2A, suggesting that another regulatory factor is required. This system may be reconstituted with other B cell specific factors, such as BSAP, to determine whether further development may occur.

It has been problematic to identify a system in which B cell development can be studied that is both physiological and easy to manipulate. Several different systems have been utilized to test the requirements of B lymphocyte commitment and differentiation. The Ba/F3 immature hematopoietic cell line used in our studies has been used for similar purposes by other laboratories. However, this cell line does not have a stable phenotype and furthermore, does not clearly resemble known lymphoid progenitors. An alternative cell culture system has been developed that consists of B cell line, 70Z/3, and a macrophage-like cell line that spontaneously developed from 70Z/3. Kee and Murre have used this system to study the function of E12 in early B cells, as discussed later in this chapter (Kee and Murre, 1998). One criticism of this method is the possible presence of B cell specific factors, from the original lineage, that may predispose the macrophage like line to differentiating into a B cell line. However, the 70Z/3 B and macrophage lines do provide a convenient in vitro system with which to test the specific biochemical functions and interactions of transcriptional activators. Alternatively, hypomorphic mutant mice may be bred together to reveal genetic interdependency. This method, commonly used in yeast genetic studies, was used to show that B cell development was dependent on a critical level of bHLH factors (Zhuang et al., 1996). We have used this approach to study the physiological functions of EBF and E2A.

Our analysis of mice lacking one Ebf and one E2a allele each, identified new genetic targets of these transcription factors, and confirmed those identified

in cell culture experiments (Chapter 4). These targets were both specific to B cells or to the lymphoid lineage: Pax5, Rag1, Rag2, mb1, λ5 and VpreB. Recent data has also suggested that EBF may regulate B29, which encodes a signaling component of the antigen receptor (Akerblad et al., 1999). B29 was only modestly affected in our assay. More importantly, we were able to observe an effect on later stages of B cell development, which was not possible in the homologous mutants that are completely lacking in the B lineage. These data suggest a temporal regulation in B cell development that is tightly controlled by a specific combination of transcription factors, such as EBF and E2A. Our experiments also led us to clone the B cell specific promoter of the Pax5 gene, with which we were able to show direct regulation of Pax5 by EBF. Binding sites for EBF, E2A and BSAP have been found together in the promoters of some B cell specific genes, such as λ5, suggesting that Pax5 may also collaborate with EBF and E2A in the regulation of B cell differentiation (Kudo et al., 1987; Yang et al., 1995, see Chapter 3).

The components of E2A, E12 and E47, appear to contribute distinctly to B lymphocyte development. Studies in which E12 or E47 expression has been reconstituted by breeding E12 or E47 transgenics to the E2A knockout, have shown that E12 seems to contribute to very early events in B lineage development (Bain et al., 1997). E12, heterologously expressed in the 70Z/3 derived macrophage cell line, upregulates the expression of both Ebf and Pax5 (Kee and Murre, 1998). E47, on the other hand, appears to be important

throughout B cell differentiation. E47 binds as a homodimer to the regulatory regions of many B cell specific genes, notably the immunoglobulin enhancer (Bain and Murre, 1998; Murre et al., 1994; Murre et al., 1989). E47 itself is specifically regulated post-transcriptionally in the B lineage. Homodimer formation, which occurs uniquely in B lymphocytes, may be critically regulated by the formation of intermolecular disulfide bonds, or by dephosphorylation which allows these disulfide bonds to form (Benezra, 1994; Shen and Kadesch, 1995; Sloan et al., 1996). This regulatory activity appears to be B cell specific, thus the nature of this regulation is of great interest. E47 is required before Pax5 in development, however, both Ebf and Pax5 are expressed very early in differentiation and may contribute to the regulation of this post-transcriptional modification of E47 (Bain et al., 1994; Lin and Grosschedl, 1995; Urbanek et al., 1994; Zhuang et al., 1994, see Chapter 4). The results of many experiments suggest that E12 may be an early step in a cascade that upregulates Pax5 and Ebf which in turn, possibly regulate the formation of the E47 homodimer, but certainly initiate transcription of a number of B cell specific genes.

The regulation of Pax5 is likely to be pivotal in the understanding of B lineage commitment and differentiation. Pax5 deficient B cells progress to a stage that is committed to the B lineage in wildtype animals (Urbanek et al., 1994). When transferred to a recipient mouse that lacks B or T cells, these "pro-B" cells can reconstitute other hematopoietic lineages, such as T lymphocytes (M. Busslinger, personal communication). Thus BSAP, encoded by Pax5, may be a B

lineage commitment factor. Our data, from experiments in mice and cell lines, show that EBF and E47 are necessary but not sufficient for normal expression of the Pax-5 gene. E12, in addition to EBF, may be sufficient for expression of Pax-5, as suggested by Kee and Murre (Kee and Murre, 1998). This hypothesis is consistent with both our in vitro and vivo observations (Chapters 3 and 4). Identification of what other factor or factors, in addition to EBF and E2A, may contribute to the activation of Pax5 will add critical information to the evolving picture of B cell commitment.

BSAP, encoded by Pax5, has been implicated in many aspects of B cell development, such as cell cycle and terminal differentiation (Busslinger and Urbanek, 1995; Usui et al., 1997; Wakatsuki et al., 1994). Intriguingly, although BSAP sites have been found in the promoters of many different genes only a subset of these genes are affected in the Pax5 knockout mice, such as Lef1, hinting at a more complex role for BSAP than originally suggested. BSAP has been reported to have negative regulatory activity as well, depending on the promoter context and the levels of BSAP available (Wallin et al., 1998). Normal Lef1 expression is dependent on BSAP, although there is no evidence that the regulation is direct (Nutt et al., 1998). LEF-1 is an activator that in other tissues has been shown to integrate extrinsic signals, specifically the Wnt signaling pathway, into a transcriptional response (Eastman and Grosschedl, 1999; Fan et al., 1998; Hsu et al., 1998; van de Wetering et al., 1997). In T cells, LEF-1 can activate transcription from the TCRα enhancer (Travis et al., 1991). We have

identified several genes in the B cell lineage whose expression is affected in the absence of LEF-1 (Chapter 5). These genes, TdT, fas, c-myc and N-myc may be important in the survival and proliferation of B cells, and in the generation of antibody diversity. Further studies are being done in the laboratory to link the function of LEF-1 with Wnt signaling in lymphocytes.

Taken together, our observations and those of other laboratories, define a hierarchy of transcriptional regulation that specifies the early events of B lineage differentiation (Fig. 6-1). EBF, E2A and BSAP are part of an intrinsic genetic program that directs the expression of B cell specific genes. LEF-1 may provide a sensor of the extracellular environment that is important in normal B cell differentiation. These connections provide a framework from which to identify new pathways and interactions. There are still many "black boxes" in B cell development that remain to be explored. Recent advances in genomics that allow the assessment of differences in gene expression between any two different B cell populations are likely to provide novel connections that will contribute to a global picture of B lymphocyte differentiation.

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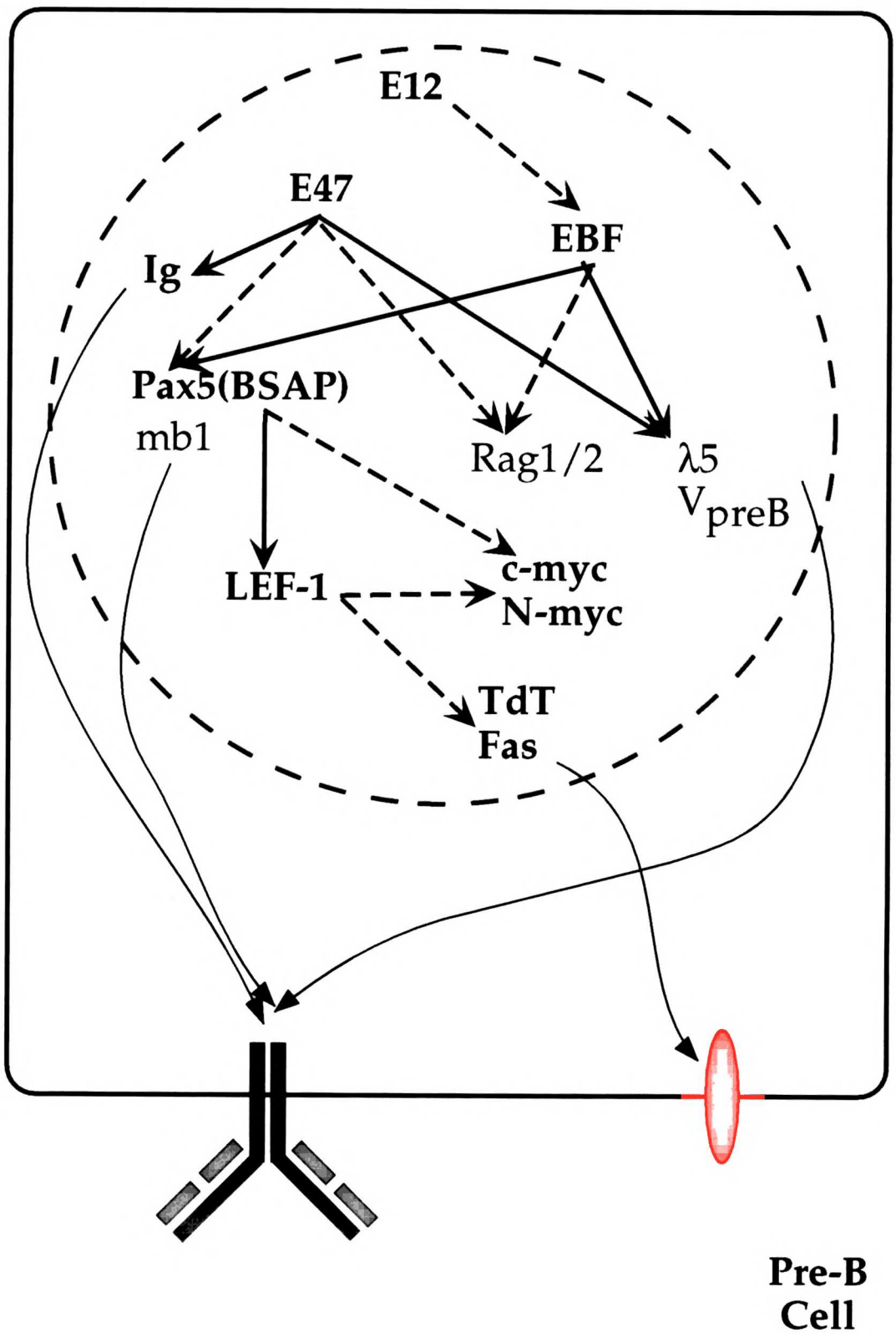
Figure Legends

Figure 6-1.

A model of transcriptional regulation in early B lymphocyte differentiation.


E12
E47
EBF
Ig
Pax5(BSAP)
mb1
Rag1/2
λ5
VpreB
LEF-1
c-myc
N-myc
TdT
Fas
Pre-B
Cell

APPENDIX I

A Rearranged Immunoglobulin Transgene Cannot Rescue Development in $Ebf^{+/-}E2a^{+/-}$ Fetal Liver Pro-B Lymphocytes

The loss of one allele of *Ebf* and one allele of *E2a* results in a marked defect in early B cell differentiation (discussed in Chapter 4). Pro-B lymphocytes from the fetal livers of *Ebf*$^{+/-}$*E2a*$^{+/-}$ mutant mice exhibit an altered gene expression profile. Expression of the *Rag1* and *Rag2* genes is significantly decreased in *Ebf*$^{+/-}$ *E2a*$^{+/-}$ pro-B cells compared to wildtype or their *Ebf*$^{+/-}$ or *E2a*$^{+/-}$ littermates. We therefore conclude that the *Rag* genes are genetic targets of EBF and E2A. The recombinase activating genes, *Rag1* and *Rag2*, play an important role in the rearrangement of immunoglobulin genes that ultimate produce a functional antigen receptor. Mice containing mutations in the *Rag1* or *Rag2* genes exhibit a complete arrest in B cell differentiation that can be alleviated by the expression of rearranged Ig transgenes (Mombaerts et al., 1992; Shinkai et al., 1992; Spanopoulou et al., 1994).

To determine whether the defect in the differentiation of *Ebf*$^{+/-}$*E2a*$^{+/-}$ pro-B cells was due to the low expression of the Rag genes, we examined whether the expression of a rearranged immunoglobulin heavy and light chain transgene (Tg^{+}) could overcome the B cell deficiency in *Ebf*$^{+/-}$*E2a*$^{+/-}$ mutant mice (Goodnow et al., 1988; Mason et al., 1992). Fetal liver cells from the offspring of a cross of *Ebf*+/-*Tg*$^{+}$ and *E2a*+/- mice were incubated with antibodies against B220, CD43, and IgM, and analyzed by flow cytometry (Fig. AI-1). In spite of the presence of the rearranged transgene, which was strongly expressed in wildtype fetal liver (middle lower panel), the *Ebf*+/-*E2a*+/-*Tg*$^{+}$ embryos contained very few pro-B cells (upper right panel). The lack of detectable transgene expression in the *Ebf*+/-*E2a*+/-*Tg*$^{+}$ mice is presumably due to the lack of pro-B cells and not due to inactivity of the transcription control elements of the transgene, which are known to be functional at an earlier stage (Strasser et al., 1990). The similarity of the phenotype in double heterozygous mice with or without the Ig transgene leads us to conclude that the B cell deficiency seen in *Ebf*+/-*E2a*+/- pro-B cell development is not due to a defect in rearrangement or expression of the immunoglobulin genes.

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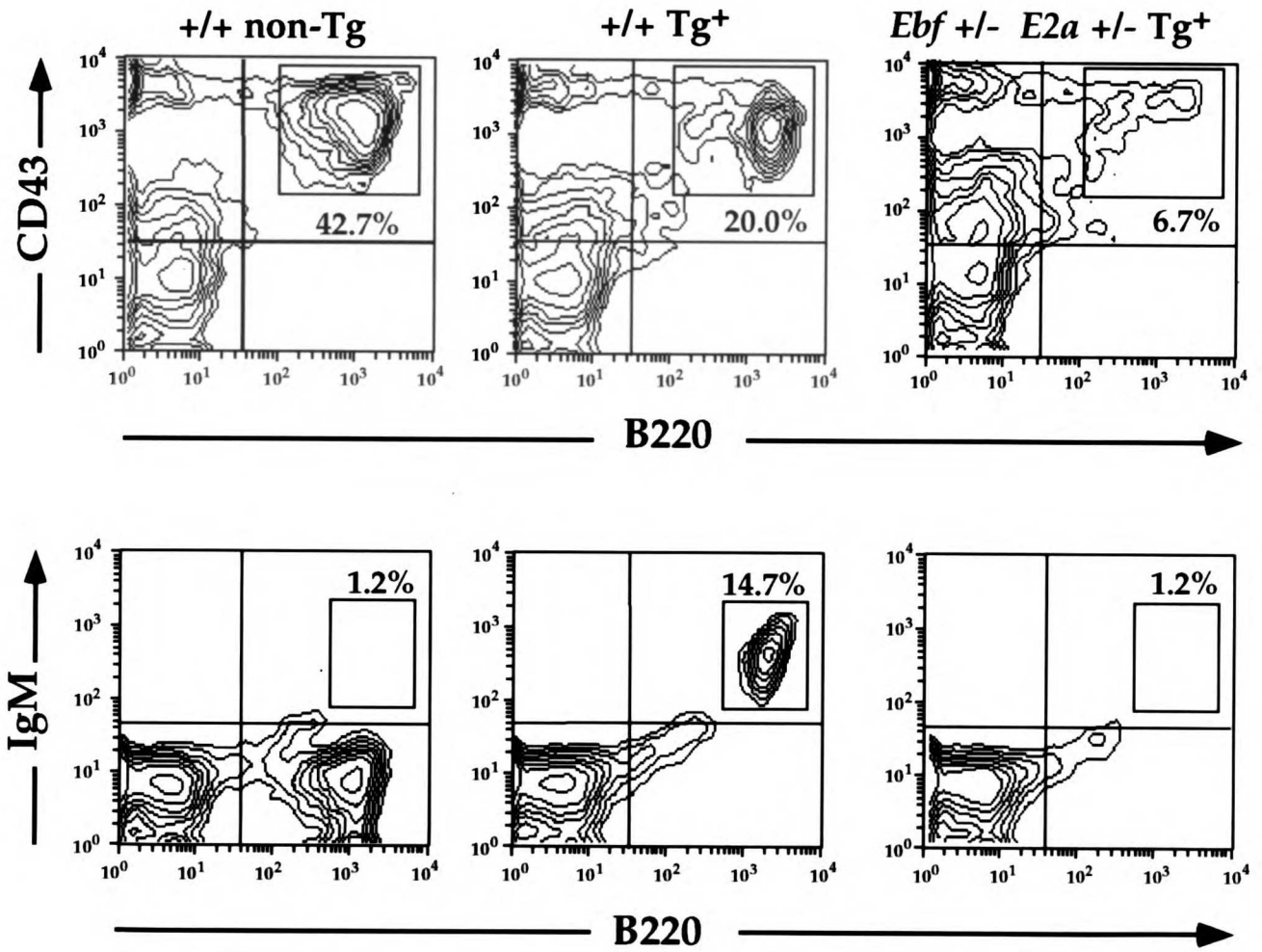
Figure AI-1.

A rearranged immunoglobulin transgene cannot rescue *Ebf+/-E2a+/-* pro-B cell development.

Fetal liver cells from E18.5 embryos were stained with anti-B220-PE, anti-CD43-biotin and anti-IgM-FITC, and subjected to flow cytometry. Non-transgenic fetal livers normally contain very low numbers of IgM^+ cells. The pro-B cell population is defined as $B220^+CD43^+$. Expression of IgM is clearly seen in wildtype animals with the transgene (Tg^+); however, the double heterozygote shows no expression most likely due to the severe reduction in the number of B cells. Numbers beside each box represent percentage of lymphocyte gated cells.


+/+ non-Tg
+/+ Tg+
Ebf +/- E2a +/- Tg+
CD43
B220
42.7%
20.0%
6.7%
IgM
B220
1.2%
14.7%
1.2%

APPENDIX II

Sequence of the Pax5 B Cell Specific Promoter

Figure AII-1

Pax5 promoter sequence (Genbank #AF148961)

Key: EBF site; TATA box; **exon 1A**

5'CGACGGCCCGGGCTGGTCCCTCCCATTCAAAAGCTCATTAAGAAAATCAAAAA

TCGAAGAAAACAAAATAAATACATTTGTCGATGCAGGACACACAAGGCACTAA

CTGAAACAGAATCAGATCATATCAAAACACAGCTCAGAAGGAGAAGCCTGGCA

TAGGACTCAAAAGGAGACAGTCTACAAGGGCAGAGTATAAAACCCACACAAA

ATCAAAACACGCAGTACAGACAACAAAACCCACCCCAGAGCTAGACCTGAAA

AAGCAGTTCCACAAAGAGGGGAGGAAAAAGCAAAACGCAAGAAACAAAAGAT

TCACGGATAATGGAAACTCTTAGGAAACAAACAACAAAACAAAACCCTAAATA

GGAAGAAAAGAAAAGCCAACAACTCCAGCCCCAGCCCAGGGTGAACTGTCAAT

TCCGAAAGAGCTTTTGATCTCCAGGCGCAGGCAGGCCTCCCCTCCGCAGCGCTG

CTTCCCGGCACCCGCGAGGCGGAAGGGCGGGAAGCGGGAGCAGGGGCCCGGA

CGCAGACGACGGTCTAAAGCTCCAGGACCGCCAAAAATCAAACCGCAGATAGG

GACCCGGACCCACAGGCTGCAGGGAGGGGTGGCACAGGGGACTACGCAGACTA

CTTGGGTGGGGGTGGGGGGACTCCCGGGAATCTACAGGCCACGTTTTCCAATTT

GTTGGCGAAAATCTGCTCCAGTGAATTTGCGATGTCCGGTGCCCACTGACCCCTC

CCCCTATCCTCGAACTCTAGGCTCTCTCCCCTAGGTCGCAGCCCAGCCTCGTGAC

CACCCCCCTCCAAAAAAAAAAAAATCAAACAAACACTCTCGGAGAGCGATTCA

CTTTCCAAAACTGCATTGTCAGTGGCCCTAGAGCGCTCACCCGCCCTGGAGAGC

CTTCCCCTGCGACCACCCCCGCAACGGGGCTCTATAGGCTTCTCGCGGCCAAAC

CCAGCCGCTTGGAATTCAGACTGGGGAGGGAGCAGAGTAGAGGAGGCTTGAGC

TCGGACGGCAGGGAGCGAGGTTGGACACCACACCTTTACCTAGTTCCTCTGCAA

Figure AII-1 (continued)

CCGATTTTCTCTCTGGAAAATCAAGTCTGGGAACTCGGGGATGCTACAAGGTCTC

TTGGCCCGAGGGCAGTGGGATTCCATTTAGGGGCCTGGGTAAGGTGTCCAGAAT

TTTTGCCTGTTCTCCGAGCCTGCTAGGATGGTTCTGCTTGGCCCGGGTGCTCCAAA

AGCAGAGGACCGGGTTTTATCAGCAGCTTTCCGCTGAACCTCTGGGCTCTGGGGT

AAAAAGGGTCGAGTTCTAAAGGGGAGATCTGGGACCTGGTGGCTGAATGGGAC

CTAGATACTGTAGTGATCTGAAGCTGCCCTGGAAGATGAGCCCAGCAGACCTAT

GGAGGTTGCAATTGAGTTGGGGGTGGGGTAGTTGGGAACCCCGAGGGCGGGCG

CCGCCCTAGGGGTGAATCTGAGGATGCTGGAGCATCCCCTGTGGTTGACAATTG

TGCTAGTACAGGGTTCAAAACCACTTGCTTGGCAGAATTGTTCTTTCTTTAAAAT

AAAAAAATGGGCCTACTGGCCCTCACAAAGCAGAGTTTACATGTAGAGCAAAA

GCGCACAGGGCCGCGACCCCCAGACACAGGTTTGGAAGCGGGTACGACCCTGT

GACTCAGGTTCTCCTTCCCCTAGGCGCGCGACTAACGCCGGGCCGGGCGCAGTC

TGAATCTTTCCTTCCCTGCCCCCAACCCCCTATAAAAGTCCAAGGCGGCACCGA

GGC**GGCATTGCTGCTCTCTGGGCCTCTTGCACACCTTCGGCCAGGCCTGGTC**